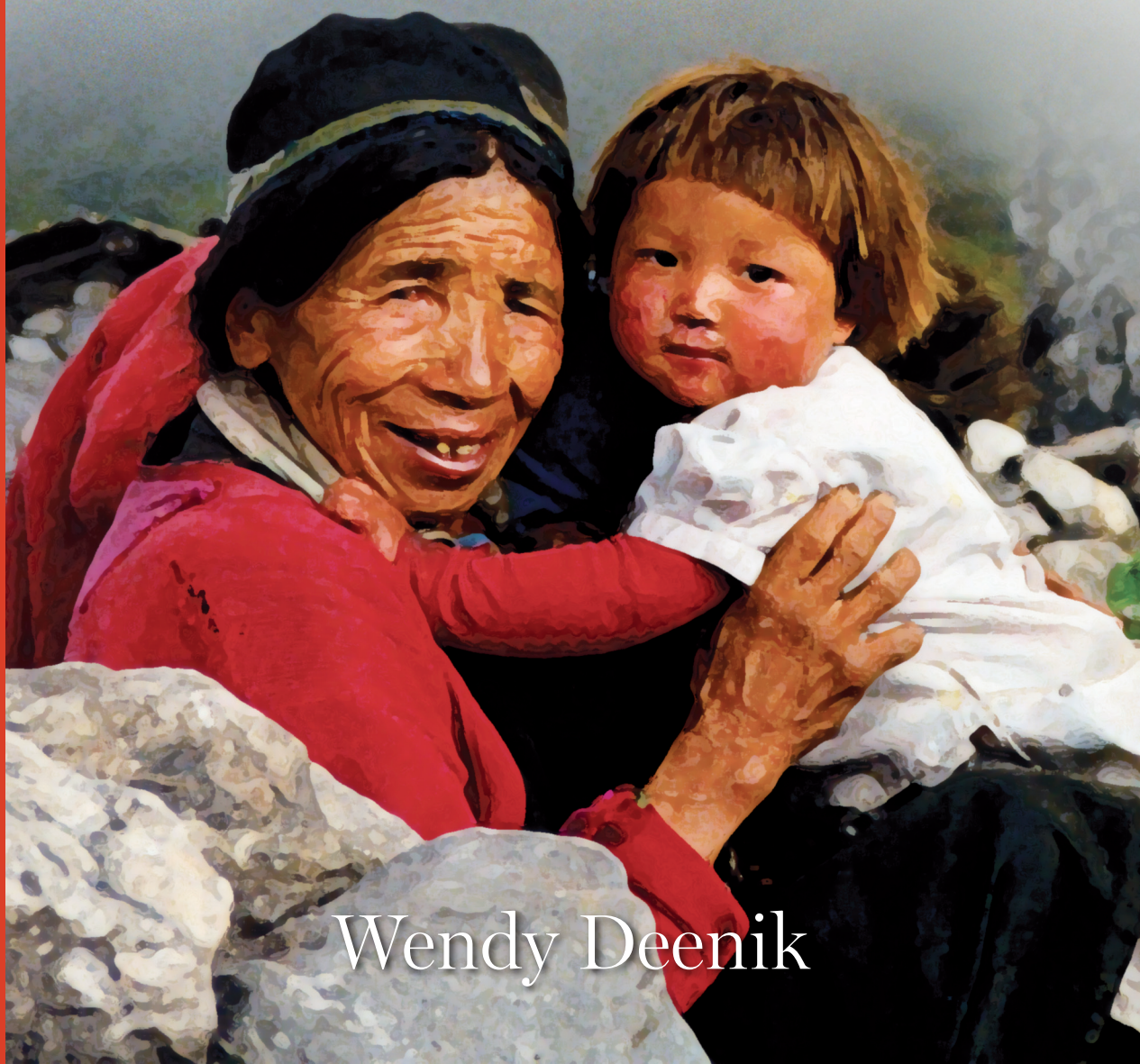
PREVENTION OF RESISTANCE IN CHRONIC MYELOID LEUKEMIA: THE ROLE OF COMBINATION THERAPY



Wendy Deenik

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Wendy Deenik © 2009

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PREVENTION OF RESISTANCE IN CHRONIC MYELOID LEUKEMIA: THE ROLE OF COMBINATION THERAPY

Preventie van resistentie in chronische myeloïde leukemie: de waarde van combinatietherapie

Proefschrift

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Wat sterk en star is, wordt afgebroken en neergeworpen.
Wat buigzaam en zacht is, zal altijd weer opkomen.
Lao Tse (zesde eeuw v. Chr.) China.

Voor mijn ouders en Kevin.

Contents and publications

1
INTRODUCTION

In part NTvH 2007;4:7-16.

1. Introduction

Chronic myeloid leukemia (CML) is a rare disease with a worldwide incidence of approximately 1-2 cases per 100,000 individuals. Chronic myeloid leukemia occurs slightly more frequently in men than in women. The median age at diagnosis is approximately 60 years, and although the incidence increases with age, it also occurs in paediatric patients. Three distinctive phases can be recognized. First, an initial chronic phase that has an average duration of 4 to 6 years without appropriate treatment. During that phase there is a gross expansion of the myeloid compartment, but the cells still retain the capacity to differentiate and function normally. Symptoms in this phase are generally mild; approximately half of the patients have no complaints, and the disease is often discovered by routine blood examination. In general, response to therapy nowadays is very favourable. The next phase is the accelerated phase that has an average duration of approximately 6 months to 1.5 years. The accelerated phase is characterized by the appearance of a certain amount of more immature cells in the blood, complete loss of response to therapy, and the occurrence of constitutional symptoms. The final stage is a transformation to an acute leukemia, the so-called blast crisis, which can be either myeloid or lymphoid in phenotype, in which immature cells (blasts) dominate and survival is measured in weeks to months. Patients with blast crisis have many complaints, and response to treatment is generally very poor.

The pathologists Bennet, Craigie and Virchow first described CML as a distinct clinical entity in 1845. The discovery in 1960 of the Philadelphia (Ph) chromosome, a minute acrocentric chromosome in the bone marrow cells of patients with CML, was a major breakthrough because it was the first demonstration of a chromosomal rearrangement being consistently linked to a specific malignancy.[1] Afterwards, Rowley and colleagues discovered that the Ph chromosome results from a unique reciprocal translocation between the long arms of chromosomes 9 and 22.[2] This translocation fuses the breakpoint cluster region (Bcr) and the Abl genes and creates the BCR-ABL oncogene.[3] The *BCR-ABL* fusion gene encodes for a protein, *BCR-ABL*-tyrosine kinase, with a constitutive activated tyrosine kinase.[4] The activation of multiple signal transduction pathways in BCR-ABL-transformed cells leads to an increased proliferation, reduced growth-factor dependence and apoptosis, and a perturbed interaction of cellular adhesion. The causal role of the BCR-ABL oncogene was established after retroviral infection of bone marrow with retrovirus coding for BCR-ABL in a mouse model that simulated a CML-like disease.[5] That finding strongly fuelled the search for a specific inhibitor of the BCR-ABL tyrosine kinase,[6] which eventually resulted in the identification of the specific inhibitor imatinib, which became a revolution in the treatment of CML and was elegantly reviewed by Deininger.[7]

Imatinib is a 2-phenylaminopyridimidine derivate that emerged as the most promising compound for clinical development following an extensive comparison of several kinase inhibitors, since it had the highest selectivity of growth inhibition of BCR-ABL-expressing cells.[6,8] Imatinib inhibits the tyrosine kinase activity by binding the kinase and stabilizing the ATP-binding configuration of the inactive form, thereby inhibiting subsequent substrate binding.[9] Imatinib inhibits proliferation and exposure to imatinib leads to apoptotic cell death. Almost all patients with CML in first chronic phase develop a complete hematologic response (CHR), and most patients also develop a complete cytogenetic response (CCR).[10,11] The probability of a CHR and a CCR is less likely in advanced disease and these responses are of short duration. In this chapter resistance to imatinib will be introduced from a clinical perspective: definition of resistance, incidence of resistance, which mechanisms may be involved, the diagnostic procedures that can be performed in case of resistance, and how to treat as well as prevent resistance. The last part will include an outline of the thesis.

Definition of resistance against imatinib

A practical clinical way to classify resistance is the moment at which resistance is detected. Primary or intrinsic resistance is defined by the lack of an initial response at a certain point of time. In contrast, secondary resistance is defined as loss of an initially achieved response. As earlier stressed by Baccarani and colleagues from the European LeukemiaNet,[12] it is important to classify the level of resistance as being hematologic, cytogenetic or molecular resistance. Primary hematologic resistance is generally defined by failure to achieve a hematologic response at 3 months or a CHR (white blood cell count $< 10 \times 10^9$/l; differential without immature granulocytes and with less than 5% basophiles; platelet count $< 450 \times 10^9$/l; non palpable spleen) at 6 months. Secondary hematologic resistance is defined as loss of an initially achieved CHR. Primary cytogenetic resistance is defined as failure to develop at least a minimal cytogenetic response after 6 months of treatment with imatinib. In addition, failure to achieve a major cytogenetic response (MCR) at 12 months or a CCR at 18 months are classified as primary cytogenetic resistance. Secondary cytogenetic resistance is defined as loss of an initially achieved cytogenetic response, which has to be confirmed by a sequent investigation. Suboptimal response implies that the patient may still have a substantial benefit from continuing imatinib treatment but that the long-term outcome is not likely to be optimal, and can also be defined at the hematologic, cytogenetic, and molecular level.[12]

Incidence of resistance

The likelihood of a response and response duration depend on the phase of the disease in which treatment with imatinib was started. Primary hematologic resistance is rare in early chronic phase CML and occurs in less than 5% of patients (Table 1).[13,14] In contrast, primary hematologic resistance occurs in a quarter of patients treated in accelerated phase and in

two-third of patients who receive initial treatment in myeloid or lymphoid blast crisis.[15-17] Primary cytogenetic resistance defined as failure to achieve a CCR at 18 months occurs more frequently and is observed in approximately 30% of patients in early chronic phase CML, 80-85% patients in accelerated phase CML, and >90% of patients in blast crisis.[13,15-17] Primary molecular resistance defined as failure to achieve a major molecular response at 24 months occurs in approximately 60-70% of patients in early chronic phase CML and virtually all patients with accelerated phase or blast crisis. Furthermore, most early chronic phase CML patients (>90%) are molecular resistant if it is defined as failure to achieve a complete molecular response at 2 years. What about loss of response or secondary resistance? Secondary hematologic resistance is observed in approximately 5-13% of patient in early chronic phase CML. On the other hand secondary cytogenetic resistance as defined as loss of CCR occurs in approximately 10% of patients in early chronic phase CML. Furthermore, loss of response or secondary resistance is much more of a problem in advanced phase CML. For example, progression-free survival (PFS) of patients in early chronic phase CML who receive initial treatment with imatinib is approximately 80-85% at 5 years.[13] In contrast, PFS is approximately 10% at 2 years in patients who receive imatinib treatment in myeloid blast crisis, and 50% at 2 years in patients in accelerated phase.[18]

Table 1. *Frequency of hematologic primary resistance and secondary resistance to imatinib by phase of CML*

Phase of CML	Primary hematologic resistance	Secondary hematologic resistance
Early chronic phase (imatinib 400 mg qd)	5%	5-13%
Late chronic phase (imatinib 400 mg qd)	5%	10-15%
Accelerated phase (imatinib 600 mg qd)	20-30%	50-60%
Myeloid blast crisis (imatinib 600 mg qd)	60-70%	85-90%

Resistance and BCR-ABL-dependence

Resistance can also be classified alternatively, by asking the question whether the malignant clone has become independent of BCR-ABL tyrosine kinase activity or if the malignant clone is still kinase dependent. One example of BCR-ABL-dependent resistance is an increased expression of BCR-ABL tyrosine kinase, whereby the BCR-ABL kinase should still be considered as the primary target of therapy. Increasing the dose of imatinib could possibly be an approach in case of such a type of resistance. In BCR-ABL independent resistance, the leukemic cells are no longer dependent on BCR-ABL for their growth, but proliferate independently from kinase activity most often due to secondary oncogenic changes, for example by clonal evolution.[19] Increasing the imatinib dose does not make

sense in these cases and alternative treatment approaches should be pursued. In order to be able to distinguish between these two forms of resistance, BCR-ABL kinase activity under adequate imatinib dosing can be followed by evaluating for example the phosphorylation status of a protein like CRKL, that is efficiently phosphorylated by BCR-ABL. Here the phosphorylation status of the CRKL protein is taken into account,[20] which is present in CML cells with uninhibited BCR-ABL in it's phosphorylated form, but in non-phosphorylated form if BCR-ABL has effectively been inhibited. Clinical progression of CML accompanied by non-phosphorylated CRKL subsequently indicates that the malignant clone has become BCR-ABL independent. By contrast, clinical progression and the presence of phosphorylated CRKL rather suggest that the malignant clone is still dependent on BCR-ABL kinase activity and that a more effective inhibition of BCR-ABL is required.

Response monitoring

It is important to quantitatively monitor the level of response to imatinib, as there is a strong correlation between the depth of the response and the probability of progression. In addition to regular checks of blood counts and bone marrow morphology to evaluate response at the hematologic level, a cytogenetic evaluation of bone marrow must be performed every 6 months until a CCR has been achieved. Cytogenetic evaluation of the bone marrow should subsequently be repeated on an annual basis. It allows the identification of clonal evolution and also allows to follow any possible loss of an earlier-achieved cytogenetic response. In addition, it enables the identification of cytogenetic aberrations in Ph-negative metaphases. Clonal evolution is defined by additional chromosomal aberrations to the Ph chromosome. These aberrations must be found in at least 2 metaphases in order to be considered a clone. If during cytogenetic evaluation an extra Ph chromosome, trisomy 8 or an isochromosome 17q is found, this is considered as a transition to accelerated phase.[19] Additional chromosomal aberrations are sometimes also found in Ph negative clones during treatment with imatinib.[19,21,22] A minority of these aberrations is associated with myeloproliferative or myelodysplastic syndromes, but generally such aberrations do not confer a worse prognosis.[22] Having achieved a CCR, responses are preferably followed by quantitative polymerase chain reaction (Q-PCR) and expressed as log reduction of *BCR-ABL* mRNA. Such molecular monitoring of *BCR-ABL* mRNA should be performed every 3 to 6 months.[23]

Minimal residual disease (MRD)

Monitoring of MRD by Q-PCR has become standard of care in the treatment of CML nowadays. While most CML patients currently develop a CCR, only a minority of patients develop a complete molecular response (CMR).[13,24] By definition, MRD monitoring refers to the quantitative detection of *BCR-ABL* mRNA copies in the range between a residual leukemic cell mass of 10^{10} to 10^{7}, which corresponds to a leukemic cell kill of, respectively 2

to 5-log. A negative PCR usually refers to a residual leukemic cell mass of less than 10^7, which implies either absence of leukemia or a residual leukemic cell mass between 10 or 10^6 cells. It indicates that a negative PCR may not be misunderstood for absence of leukemia or cure. This has become clearly evident in patients, who stopped imatinib after having achieved PCR-negativity and who developed a relapse of their CML. Using very sensitive PCR-techniques, persistent residual disease is frequently found in patients with a CMR during imatinib therapy, while patients that have undergone an allogeneic stem cell transplantation (allo-SCT) usually lack residual disease even by very sensitive PCR techniques.[25,26]

Residual disease is often located in the stem cell compartment. Non-dividing ("quiescent") Ph^+ stem cells have been found in CML patients with good responses that have retained the possibility for proliferation.[27] Imatinib possibly delays the proliferation of these malignant stem cells, but does not induce an apoptosis, as occurs in more differentiated cells.[28] Why are these malignant stem cells less sensitive to treatment with imatinib? A number of possibilities have been suggested:

- the intracellular concentration of imatinib could be lower in stem cells than more differentiated cells, due to increased expression of, for instance, P-glycoprotein (P-gp), that actively pumps imatinib out of the stem cell.[29]
- a possible stronger expression of *BCR-ABL* mRNA in stem cells than in the more differentiated cells.[30]
- point mutations in the kinase domain of BCR-ABL in stem cells, as was found in patients with a CCR.[31] Such point mutations may possibly contribute to the survival of these stem cells.

All these possible explanations for the persistence of a Ph^+ stem cell compartment are currently receiving a great deal of attention and new approaches to prevent resistance currently particularly focus on the Ph^+ stem cell compartment.

2. Mechanisms of resistance

The following mechanisms have been described to contribute to resistance to imatinib.

A. Mutations in the *BCR-ABL* kinase domain

Mutations in the *BCR-ABL* kinase domain occur in 50 – 90% of the patients with secondary resistance. Point mutations often lead to amino acid substitutions that impair the binding of imatinib to the ATP binding site. Point mutations in the kinase domain may even be present before treatment with imatinib has been initiated. Mutations can be subdivided according to the position of amino acid substitution within the ABL kinase protein. In this way, 4 regions can be distinguished. First of all, mutations within the so-called "P-loop". The "P-loop" is a strongly preserved region, between the amino acids at positions 244 and 255, and is responsible for the phosphate binding. In particular, point mutations within this region are associated with a bad prognosis.[32] The second region is located at the level of the amino acid threonine at position 315. This amino acid plays an important role in the imatinib bond by the formation of a hydrogen bridge.[20] Substitution of this amino acid threonine with isoleucine as a consequence of a nucleotide change C-T, is associated with a complete insensitivity to imatinib. An other mutation in this region that is rarely found during treatment with imatinib, is the C-G mutation at nucleotide 1098, which results in the phenylalanine -leucine substitution at 317 (F317L). That mutation is associated with a somewhat reduced susceptibility to imatinib. The third region concerns the catalytic domain that is located between the amino acids at positions 351 and 355. The fourth region is the activation-loop or A-loop, which is located between the amino acids at positions 381 to 402. Point mutations can also be found outside these 4 regions. The clinical implications of the latter mutations are not very well known, due to their rare occurrence. There is a great difference in susceptibility to imatinib between the different point mutations, varying from none, a minimal increase of the inhibiting concentration, to a complete resistance to imatinib.[12,33] For example a M351T mutation, which confers only low-level resistance to imatinib, may respond to imatinib dose-escalation. On the other hand, some P-loop mutations (G250E, Y253H, E255K) confer a high-level of resistance to imatinib and nilotinib, but can be sensitive to treatment with dasatinib. In contrast to the V299L mutation, which is associated with resistance to dasatinib, but sensitive to imatinib and nilotinib. As opposed to the T315I mutation, which confers resistance against all three tyrosine kinase inhibitors. Collectively, these results indicate that increasing the dose of imatinib or offering an alternative tyrosine kinase inhibitor is often depending on the mutation detected. However, other mechanisms of resistance may operate, and as a consequence response on therapy in patients with the same mutation can be variable.[34]

B. *BCR-ABL* gene amplification and overexpression

Increased expression of the BCR-ABL oncogene and subsequent increased tyrosine kinase activity may confer resistance, but the leukemic clone may still be susceptible to kinase inhibition by imatinib. Increased expression of BCR-ABL can be the result of an additional Ph-chromosome or may be due to changes in transcription and translation of the oncogene. While point mutations in the kinase domain are the most frequent cause of resistance, increased expression of BCR-ABL also occurs frequently, most often as a mechanism of secondary resistance in transformed CML.[19] Usually, the clone is still sensitive to imatinib. For that reason, increasing the dose imatinib is the first step to be taken.

C. Clonal evolution

Clonal evolution is defined by the occurrence of additional chromosomal aberrations within the Ph^+ clone. Usually, malignant cell growth and proliferation have become independent of BCR-ABL tyrosine kinase activity, as outlined above. Clonal evolution is generally associated with a greater chance of disease progression, especially when it occurs in patients with a complete hematologic or even a major cytogenetic response.[19] Cytogenetic monitoring of patients with a response to imatinib, therefore, remains strongly advocated. An alternative tyrosine kinase inhibitor whether or not followed by an allo-SCT, is usually the first option to be considered for such patients.

D. Multidrug resistance

Multidrug resistance (MDR), defined by cross-resistance to a number of chemotherapeutic agents, usually occurs after earlier exposure to chemotherapy. The most well known mechanism of MDR is overexpression of P-gp, which is encoded by the MDR-1 gene. P-glycoprotein is an efflux pump that actively eliminates chemotherapeutic agents such as for example anthracyclins. An increased expression in Ph^+ hematopoietic cells may lead to the active removal of imatinib and subsequent reduced intracellular concentration.[35] That mechanism of resistance has been firmly demonstrated in vitro,[36,37] but clinical reports are rare, although a recent report from France suggested an association between molecular response and MDR-polymorphisms in CML.[38] Further clinical studies are required to evaluate the importance of this mechanism in primary and secondary resistance.

E. Metabolism

An increased plasma concentration of the α1-acid glycoprotein has also been put forward as a possible mechanism of resistance.[39] The α1-acid glycoprotein has been shown to bind imatinib in plasma. Thereby, it may increase the elimination of imatinib and reduce the effective concentration of the drug. Yet, the clinical relevance of this mechanism of resistance is unclear.

3. Alternative treatment possibilities

Nowadays there are different possibilities for second-line or third-line therapy, when resistance to imatinib has occurred. Before discussing strategies to be followed, first the different therapeutic possibilities are presented.

A. Allogeneic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation is still the only treatment modality, that has been proven to cure patients with CML. While allo-SCT was the primary treatment option before the introduction of imatinib, the application of allo-SCT has undergone an enormous reduction. Treatment related morbidity and mortality associated with allo-SCT hamper the applicability of this treatment approach and currently only resistant patients with CML in chronic phase without a favourable response to tyrosine kinase inhibitor therapy qualify for transplantation. In addition, allo-SCT has still a place in transformed CML, as outcome of accelerated CML and blast crisis are still unsatisfactory despite the introduction of effective tyrosine kinase inhibitors. An important development in the field of allo-SCT is the introduction of non-myeloablative conditioning, which is associated with a significantly lower morbidity and mortality.[40] Risk factors for treatment related mortality (TRM) after a transplant include a longer interval after diagnosis to transplant, an advanced disease stage, older age, and a female donor for a male patient. Together these risk factors constitute the Gratwohl score[41] that can be used to estimate the risk of TRM. Despite the introduction of non-myeloablative conditioning and the improved identification of patients at low-risk for TRM, allo-SCT is still predominantly applied in second- or third-line. Another important development in the field of allo-SCT is infusion of donor lymphocytes many months after stem cell transplantation, which has been demonstrated to be associated with less graft versus host disease and thereby less TRM. Especially in CML, the anti-tumor effect of donor lymphocytes against the malignant hematopoietic tissue of the recipient is particularly strong.[42] Patients with residual disease or with a relapse after allo-SCT may still achieve a CMR by the infusion of donor lymphocytes, which is then followed by a particularly favourable disease-free survival.

B. New tyrosine kinase inhibitors

- Dasatinib (BMS-354825) is a combined ABL and SRC kinase inhibitor, that may also inhibit a number of other kinases.[43] It appeared from in vitro studies with Ph^+ cell lines that the cytotoxicity of dasatinib is 325 times more powerful than imatinib. It also appeared that dasatinib may maintain it's efficacy against Ph^+ cells harboring point mutations. Dasatinib has been approved for second-line treatment of patients with CML that are resistant or intolerant to imatinib. Dasatinib is usually well tolerated and is

effective in all phases of CML. With the exception of T315I, dasatinib is also effective in patients with resistant CML as a consequence of point mutations in the kinase domain. Results of a phase II study in imatinib-resistant or imatinib-intolerant CML included a CHR in 91% of patients, a MCR 59% of patients, and a CCR 49% of patients.[44] Fifteen-month PFS and OS were 90% and 96%, respectively in that study.

The most frequent side effects besides myelosuppression are: diarrhea, rashes, nausea and peripheral edema. However, some patients may experience pleural effusion, which sometimes necessitates the interruption and/or stopping of dasatinib. Currently, studies are being conducted comparing imatinib and dasatinib in first-line treatment of CML.

- Nilotinib (AMN107) structurally resembles imatinib and is a very selective ABL inhibitor.[43] In order to bind to ABL; nilotinib is also dependent on the inactive form of the kinase protein. The affinity of nilotinib for the ABL kinase is, however, much stronger. The efficacy of nilotinib appeared 10 - 50 times more powerful than imatinib and nilotinib appeared also effective against many kinase domain mutations, except the T315I mutation. Results of a phase II study in imatinib-resistant or -intolerant CML included a CHR in 74% of the patients, a MCR in 48% of the patients, and a CCR in 31% of the patients.[45] The estimated overall survival at 12 months was 95%. Also nilotinib has been approved for second-line treatment of patients with CML. Generally, nilotinib is well tolerated. Side effects that are frequently found are myelosuppression, rashes and hyperbilirubinemia. Clinical studies comparing nilotinib and imatinib are currently being conducted.
- Currently, a number of other kinase inhibitors are being developed,[46] but clinical studies with these newer inhibitors are scarce.

C. Combination therapy

With interferon alfa (IFN-α) and cytarabine being the cornerstones of therapy before the introduction of imatinib, different investigators rapidly became interested in the combined activity of the older agents with imatinib following it's approval. The rationale for combination therapy lies within the prevention of resistance and the pursuit of an improved response and prolonged response duration. The ability of imatinib to overcome resistance to apoptosis by various chemotherapeutics further added to the development of new studies exploring combination therapy. It was also suggested that combination therapy might possibly eliminate the Ph^+ stem cell compartment more effectively. Different in vitro studies suggested synergism between imatinib and chemotherapeutic agents, including cytarabine.[47] Feasibility studies of the combination of IFN-α and imatinib and the combination of cytarabine and imatinib have shown that combination therapy was feasible and preliminary evaluation of efficacy showed at least comparable response percentages.[48-50] In the second and third chapter of this thesis the feasibility and efficacy is described of the HOVON-51

study, in which combination therapy consisting of different dosages of imatinib (200 mg, 400 mg, 600 mg or 800 mg) and cytarabine (200 mg/m^2 or 1000 mg/m^2) were given to newly diagnosed patients with CML.

D. Interferon alfa, hydroxycarbamide

The conventional agents hydroxycarbamide and IFN-α are currently rarely applied as, apart from imatinib, also the newer second line kinase inhibitors offer better possibilities in the treatment of CML. However, intolerance of treatment and special circumstances such as pregnancies may necessitate the use of either hydroxycarbamide or IFN-α. Response percentages and duration of the response are probably not different from results extensively reported in the past for patients with CML in the chronic phase.[51]

4. Treatment guidelines for resistance

Imatinib has revolutionized treatment of CML and is associated with high response rates and excellent survival.[14] Nevertheless, resistance is of concern especially in transformed CML. Furthermore, response percentages in first chronic phase CML are high, but still a considerable percentage of patients is in need of second-line treatment due to progression, resistance, or intolerance of treatment. A recent report form the Hammersmith Hospital estimated that approximately 25% of patients who started on imatinib for chronic phase CML required a change of therapy after a median follow-up of 3-4 years.[13] Moreover, while cytogenetic resistance is rare, resistance at the molecular level is common with approximately 90-95% of patients showing signs of MRD by Q-PCR if treated with a standard dose of 400 mg imatinib daily. Therefore, careful monitoring and adaptation of therapy based on current guidelines remains important.

A. Guidelines for resistance in chronic phase CML

As described above, careful hematologic, cytogenetic, and molecular monitoring is very important. Therapy must considered insufficient and is to be adapted if there is no hematologic response at 3 months, no minimal cytogenetic response at 6 months, no MCR at 12 months, and no CCR at 18 months.[12] Failure to imatinib therapy also includes loss of CHR, loss of CCR or a mutation conferring a high level of resistance to imatinib. Also in case of suboptimal response, adaptation of the treatment must be considered. Suboptimal responses include no CHR at 3 months, no MCR at 6 months, no CCR at 12 months, and no MMR at 18 months. Furthermore, at any time additional chromosomal abnormalities in the Ph$^+$ cells, loss of MMR or mutations conferring a low level of resistance to imatinib. In case of a suboptimal response to imatinib, first the dose of imatinib must be increased to 600 - 800 mg. This recommendation is based on the earlier demonstrated dose response relationship

of imatinib and the preserved sensitivity to imatinib in most cases of suboptimal response.[52] Alternative therapy must be considered if an increase of imatinib does not induce the required response within 3 - 6 months. Insufficient responses may necessitate an immediate change to an alternative kinase inhibitor. Currently, either dasatinib or nilotinib may be considered then. Choices for a particular second-line kinase inhibitor may depend on whether or not a mutation was detected and which type of mutation; whether clonal evolution is present; to what extent therapy was tolerated; and whether the patient also suffers from comorbidities. For example, in cases of pulmonary comorbidity, nilotinib may be preferred, while in case of cardiovascular disease and diabetes dasatinib may be preferred. Choices of second-line kinase inhibitor and type of mutation were elaborately described by Weisberg et al.[53] In general, nilotinib is preferred if the malignant clone is still dependent from BCR-ABL kinase activity and no P-loop mutations with high level of resistance to imatinib and/or cytogenetic evolution is present, all other cases may require dasatinib as the strongest and broadest kinase inhibitor. Response to second-line treatment is subsequently evaluated again at regular intervals by blood and marrow morphology, cytogenetics, and Q-PCR. Favorable responses are to be followed by continuation of second-line treatment, which is currently associated with excellent survival.[44,54] while insufficient responses may require a careful weighing of the possibilities of an allogeneic hematopoietic stem cell transplantation.

B. Guidelines for resistance during acceleration phase or blast crisis

In general, blast crisis requires an intensive approach such as currently applied in the management of poor-risk acute leukemia, including the combination of a tyrosine kinase inhibitor and chemotherapy, preferably followed by an allo-SCT. First, it is important to obtain a hematologic remission, a so-called second chronic phase. A hematologic response can be achieved with imatinib monotherapy in approximately 30% of the patients in blast crisis,[18] Results with intensive chemotherapy without a kinase inhibitor may be comparable, but combination therapy consisting of imatinib, cytarabine and idarubicine or daunorubicine have yielded better results, as was reported in small groups of patients with a myeloid blast crisis.[55,56] Blast crisis characterized by an increase of lymphoblasts usually requires an induction schedule base on what is currently applied in acute lymphoblastic leukemia, including corticosteroids, vincristine and anthracyclins in combination with imatinib, and intrathecal treatment. Blast crisis that develops in patients already using imatinib, obviously requires the use of a second-line kinase inhibitor to be combined with chemotherapy. As indicated above, dasatinib may be the preferred inhibitor as blast crisis is usually associated with clonal evolution and/or mutations of the *BCR-ABL* kinase domain. Induction combination therapy resulting in a hematologic response is to be followed by consolidation therapy by allo-SCT using either sibling or alternative donors. Also cytogenetic or even molecular responses are preferably consolidated with an allo-SCT, because the

relapse rate is high despite continuation of second-line kinase inhibitor therapy.[57] Likewise, accelerated phase CML phase, requires reinduction by dasatinib or nilotinib to be followed by consolidation using an allo-SCT. Only if a molecular response is obtained, the alternative inhibitor may possibly be continued under accurate monitoring (hematologic, cytogenetic, and molecular). If, during follow-up, *BCR-ABL* mRNA levels increase, an allo-SCT must be aimed for as soon as possible.

C. Guidelines for the prevention of resistance

Prevention of resistance may be pursued by a variety of approaches. Given the high percentages of complete hematologic and cytogenetic responses, especially prevention of molecular resistance is currently an issue. First, a higher starting dose of imatinib as suggested by data of the M.D. Anderson may result in a higher molecular response rate.[58] Why would it make sense to strive for a better molecular response? While patients achieving a CCR show excellent PFS, patients with major molecular responses (MMR) show even better PFS with virtual absence of progression as was reported by Hughes et al.[24] and also observed by the Italian cooperative group.[59,60] Recent reports using either dasatinib or nilotinib as first line treatment may suggest higher molecular response rates by use of these stronger kinase inhibitors,[61,62] but long-term PFS figures are currently not available with these approaches. Another possibility to rapidly achieve a better molecular response is combination therapy by using both a kinase inhibitor and a chemotherapeutic agent like cytarabine or IFN-α. A rapid reduction of the leukemic cell burden below the detection limits of Q-PCR might be associated with a reduced probability to develop point mutations and clonal evolution and thereby result in improved PFS. That rationale underlies the approaches, which were studied in the present thesis.

5. Outline of this thesis

Until the year 2000, therapeutic options for patients with CML were limited and included IFN-α, low-dose cytarabine, and/or allogeneic hematopoietic stem cell transplantation. Imatinib has revolutionized the treatment of patients with CML since it's first introduction in 1998. Hematologic and cytogenetic resistances are currently very rare in first chronic phase, but most patients treated with a dose of 400 mg imatinib continue to experience residual disease at the molecular level, which can be considered as molecular resistance.

The first study (HOVON-38) described in this thesis stems form the pre-imatinib era and addressed the question whether intensified cytarabine followed by IFN-α would be superior to low-dose cytarabine combined with IFN-α. Long-term results of that study are described in Chapter 2. They illustrate the enormous progress made in the years following the introduction of imatinib, which was facilitated in The Netherlands and Belgium by the conduct of the HOVON-51 study. That study (HOVON-51) addressed the question whether combination therapy of imatinib with cytarabine would be feasible (Chapter 3) and would improve outcome (Chapter 4) in CML patients in early chronic phase. Parameters possibly associated with better molecular response were subsequently investigated. The question arose, whether the 3 most relevant multidrug resistance gene (MDR1) polymorphisms were associated with molecular response to combination therapy. This question was addressed in a subgroup of patients in the HOVON-51 study who received high-dose (800 mg) imatinib (Chapter 5). Cytogenetic resistance occurs more rarely, but molecular resistance to imatinib is abundant and has been suggested to originate in the Ph^+ stem cell compartment. Therefore, new approaches to prevent and treat resistance currently focus on the elimination of Ph^+ stem cells in particular. In Chapter 6, a flowcytometric approach is described, that identifies malignant stem cells and discriminates them from their normal counterparts. Currently, 2 alternative kinase inhibitors (dasatinib; nilotinib) are available, that were approved for second-line treatment. In Chapter 7, treatment results are described of an upfront approach using the combination of dasatinib and chemotherapy in a patient with a NUP214-ABL1 -positive acute lymphoblastic leukemia. The place of dasatinib in this particular type of leukemia is discussed. Finally, the results of our studies are discussed more in general with emphasis on the role of cytarabine, the current place of molecular monitoring in CML, and, lastly, the role of the new tyrosine kinase inhibitors in imatinib-resistant CML.

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HIGH-VS LOW-DOSE CYTARABINE COMBINED WITH INTERFERON ALFA IN PATIENTS WITH FIRST CHRONIC PHASE CHRONIC MYELOID LEUKEMIA. A PROSPECTIVE RANDOMIZED PHASE III STUDY.

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Abstract

A prospective randomized phase III study was performed to evaluate whether intensified cytarabine would induce a higher response rate and longer event-free interval as compared to low-dose cytarabine in chronic myeloid leukemia (CML). One hundred and eighteen patients with CML in early chronic phase entered the study. Twenty-eight out of 32 patients assigned to group A received two cycles of a combination of intensified cytarabine and idarubicin followed by interferon alfa (IFN-α) maintenance, 28 patients in group B received standard treatment by a combination of low-dose cytarabine and IFN-α. Forty-nine patients with an human leukocyte antigen-identical sibling donor proceeded to an allogeneic stem cell transplantation (allo-SCT) and nine patients were excluded from the analysis. Hematologic response was observed in 97% of the patients in group A versus 86% of the patients in group B during the first year of treatment. In group A, 16 patients (50%) achieved a major cytogenetic response, which compared to seven patients (25%) with a major cytogenetic response in group B. With a median follow-up of 58 months (range: 34 - 76), event-free survival (EFS) was not significantly different between arms A and B. The estimated 5-year survival rate was 56% in the intensified arm and 77% in the low-dose arm (P=.05). Recipients of allo-SCT showed a 5-year estimated survival rate of 55%. Although intensified cytarabine induced a higher initial percentage of major and complete cytogenetic responses, responses were not sustained by IFN-α maintenance therapy.

Introduction

Chronic myeloid leukemia (CML) is a malignant hematopoietic stem cell disorder, characterized by a unique reciprocal translocation between the long arms of chromosomes 9 and 22 resulting in an aberrant chromosome 22, also known as the Philadelphia chromosome.[1] The result of the translocation is the generation of a BCR-ABL fusion gene which encodes for a chimeric protein (p210) with dysregulated tyrosine-kinase activity sufficient for leukemogenesis in mice.[2]

Before the introduction of imatinib, which is currently considered standard first-line treatment, patients with CML in first chronic phase were preferably treated with a combination of interferon alfa (IFN-α) and low-dose cytarabine, as the addition of cytarabine had been shown to result in a higher response rate and enhanced survival in a randomized study.[3] We performed a pilot study in 19 patients exploring the feasibility of intensified cytarabine in patients with CML,[4] as cytarabine may exert a dose-response effect in myeloid leukemias.[5,6] This schedule of intensified cytarabine combined with idarubicin proved feasible in these patients, while an encouraging major cytogenetic response rate of 60% was observed. In contrast to acute myeloid leukemia (AML) intensive chemotherapy has received relatively little attention in CML so far, as the potential morbidity and mortality was considered a major hurdle.

In studies exploring the efficacy of intensive chemotherapy a major cytogenetic response was observed in 30 – 60% of patients,[7-10] but these responses proved transient without maintenance therapy. Given the dose-response effect of cytarabine in other myeloid leukemias, e.g. AML, we wished to address the question whether intensified cytarabine based chemotherapy followed by IFN-α maintenance would be superior to low-dose cytarabine combined with IFN-α.

Material and methods

Patients with CML were eligible for randomization if they were between 16 and 65 years of age, in first chronic phase (≤ 6 months after diagnosis), WHO performance scale ≤ 2, if cytogenetic evaluation showed the Philadelphia chromosome, or the BCR-ABL oncogene was shown by polymerase chain reaction (PCR). Patients with features of accelerated phase or blastic phase were not eligible, nor were those with severe concomitant illness as severe hepatic or renal dysfunction, severe cardiac, pulmonary or neurological disease, human immunodeficiency virus infection (HIV), prior malignancies except stage I cervix carcinoma and basocellular carcinoma, and pregnant or lactating women. Patients ≤ 55 years of age with an HLA matched sibling donor were not randomized and proceeded directly to allogeneic stem cell transplantation (allo-SCT). Written informed consent was obtained from all patients.

Study design and treatment

Initially, treatment with hydroxyurea was allowed to control the white blood cell count (WBC). In this period HLA-typing of the patients and available siblings were performed. Patients without an HLA-matched sibling donor were randomized between two groups. Randomization was stratified by center, by age (≤ 60 vs > 60 years) and Sokal risk score (<0.8 vs 0.8-1.2 vs >1.2). The high-dose arm A consisted of two courses of intensified cytarabine based chemotherapy. The first cycle consisted of cytarabine 200 mg/m² (days 1-7) combined with idarubicin 12 mg/m² (days 1,2). The second course consisted of high-dose cytarabine 3 g/m² x 2 (days 1,3,5 and 7). The dose of cytarabine was reduced to 1 g/m² in patients above 60 years of age. The low-dose cytarabine was given to patients assigned to arm B of the study. They received IFN-α 3-9 x 10^6 Units (U) daily in combination with cytarabine 20 mg/m² s.c., days 1-10, administered every four weeks. If the WBC reached a nadir of 1 x 10^9/l or the platelet count was below 50 x 10^9/l, the dose of cytarabine was reduced to 15 mg/m² for 10 days in the subsequent cycles. A similar dose adaptation was performed if the WBC and platelet count had not recovered to, respectively, 3 x 10^9/l and 100 x 10^9/l, within 5 weeks after start of cytarabine. Combination therapy was given for 1 year, but could be prolonged for a maximum of 2 years in patients achieving cytogenetic response. Cytarabine was discontinued in case of a complete cytogenetic response documented twice at least 3 months apart. Both therapies were followed by IFN-α maintenance therapy until disease progression or intolerance. Maintenance treatment with IFN-α was started at a dose of 3 x 10^6 U daily. After one week the dose was increased to 4.5–6 x 10^6 U daily. Thereafter, the highest tolerable dosage was given, aiming at a dose of 4.5–9 x 10^6 U daily. In case of common toxicity criteria (CTC) grade 3 (severe) or grade 4 (life threatening) toxicity, therapy was interrupted and resumed at a lower dose after recovery of toxicity. In case of hematologic toxicity, therapy was temporarily withheld if the WBC was below 2 x 10^9/l or the platelet count dropped below 25 x 10^9/l. When the platelet count measured between 25 and 50 x 10^9/l the dose was reduced with 50%. The concurrent administration of hydroxyurea was allowed only when the WBC exceeded 10 x 10^9/l despite adequate IFN-α treatment or above 5 x 10^9/l in the presence of CTC grade 3 - 4 toxicity, in patients not receiving low-dose cytarabine.

End points

Event-free survival (EFS) was defined as the time from randomization until either no cytogenetic response achieved at 12 months, or complete loss of any cytogenetic response thereafter (documented twice), accelerated phase, blastic phase, or death, whichever came first. Overall survival (OS) was calculated from the date of registration until death from any cause. Patients still alive were censored at the time of last contact. The criteria for a complete hematologic response were normalization of the WBC (<10 x 10^9/l) with no immature forms, a platelet count (<450 x 10^9/l) and disappearance of clinical signs and symptoms (including splenomegaly) and for failure a WBC >20 x 10^9/l. A partial hematologic response was defined

by a WBC between 10 and 20 x 10^9/l or a WBC <10 x 10^9/l but with >5% immature cells or platelets >450 x 10^9/l or a palpable spleen. Any hematologic response is the sum of complete and partial hematologic response. Cytogenetic analysis was performed every six months and classified as absent (100% Ph chromosome positive metaphases), minor (between 35 – 99% Ph chromosome positive metaphases), partial (<35% Ph chromosome positive metaphases) and complete (elimination of Ph chromosome positive metaphases).

Statistics

The primary objective of the study was to evaluate the effect of intensified cytarabine preceding IFN-α maintenance compared to low-dose cytarabine combined with and followed by IFN-α on EFS. In order to detect with an 80% power an increase in 5-year EFS from 32% to 50% (2-sided significance level α=.05) and assuming an accrual of four years and an additional follow-up of two years, 230 patients had to be randomized. Secondary end points were OS, hematologic and cytogenetic response between the two treatment arms, as well as side effects and infections of the different treatment regimens. Patient characteristics were compared between patient groups with the Pearson's χ^2 test in case of discrete variables, or the Kruskal-Wallis test in case of continuous variables. Hematologic and cytogenetic response rates were compared using Pearson's χ^2 test or Fisher's exact test, whichever appropriate. Event-free survival and OS were estimated by the actuarial Kaplan-Meier method, and 95% confidence intervals (CI) were calculated. Kaplan-Meier curves were constructed to illustrate survival, and compared between patient groups using the log-rank test. Side effects and infections were scored according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) and summarized using descriptive statistics. All reported *P*-values are 2 sided, and a significance level α=.05 was used.

Results

From February 1998 to February 2001, 118 patients entered the study. The study was closed prematurely after patient accrual ceased due to the introduction of imatinib mesylate. Sixty-three patients were randomized, 34 to arm A and 29 to arm B. However, two patients in arm A refused intensive treatment, were lost to follow-up since, and have therefore been excluded from the analysis. One patient in arm B had an allogeneic donor, and was therefore excluded from arm B, and included in the allo-SCT group. A total of 49 patients received an allo-SCT in first chronic phase. The seven remaining patients were excluded: one patient refused randomization and was lost to follow-up, while in six patients the planned allo-SCT was cancelled, or performed in accelerated phase or blastic crisis. Results are therefore presented for 32 patients in arm A, 28 in arm B, and for 49 patients who received an allo-SCT in first chronic phase. The analysis reported here was performed with a median follow-

up of patients still alive of 58 months. Patient baseline characteristics were in general not different between the three groups (Table 1), except that patients in the allo-SCT group were somewhat younger, which is a direct consequence of the lower age limit for allo-SCT in this trial. Twenty-eight patients in group A received the two scheduled courses of intensified chemotherapy and two patients received only one course of chemotherapy. Two patients received IFN-α in combination with low-dose cytarabine instead of high-dose chemotherapy. Twenty-six patients in group B received a median of 12 courses of low-dose cytarabine (range: 2 – 31). Two patients received IFN-α only, without addition of low-dose cytarabine as a result of early toxicity. The median IFN-α dose was 3.3 x 10^6 U (range: 0.7-8.4) daily during 11.6 months (range: 0.5–47.6) in group A and 4.9 x 10^6 U (range: 1.6-9.1) daily (*P*=.03) during 15.2 months (range: 2.3–60.9) in group B.

Table 1. *Baseline characteristics of the patients*

Characteristic	High-dose cytarabine	Low-dose cytarabine	Allogeneic transplantation
	(N=32)	(N=28)	(N=49)
Age at diagnosis (yr)			
median	45	52	43
range	22-64	24-65	17-55
Sex – no			
male	20	18	33
female	12	10	16
Spleen size (cm below mid-left costal margin)			
median	4	1	5
range	0-16	0-20	0-20
Platelets (x 10^9/l)			
median	460	384	496
range	90-1113	116-3400	30-1358
White cell count (x 10^9/l)			
median	148	77	175
range	5-446	4-323	12-402
Blasts in peripheral blood (%)			
median	2	1	2
range	0-17	0-9	0-15
Sokal risk group			
low <0.8	10	9	18
intermediate 0.8-1.2	12	10	14
high >1.2	8	8	12
unknown	2	1	5
Initial Hydroxyurea treatment (wks)			
median	10	8	
range	2-60	2-20	

Hematologic and cytogenetic responses, duration and survival

The cumulative incidence of a complete hematologic response was 75% in group A and 75% in group B and of a partial hematologic response 22% and 11%, respectively, in the first year of treatment (Table 2). Best cytogenetic response in the first year included a major cytogenetic response in 16 patients (50%) in group A, including six patients with a complete cytogenetic response versus seven patients (25%) in group B (*P*=.06) including one patient with a complete cytogenetic response (*P*=.11). Overall, any cytogenetic response was observed in group A in 84% compared with 57% in group B (*P*=.02).

Event-free survival measured 61% at 12 months and 23% at 36 months in patients receiving intensified cytarabine followed by IFN-α maintenance. By comparison, patients treated with standard low-dose cytarabine and IFN-α showed EFS of 54% and 37% at 12 and 36 months, respectively, (*P*=.83). Results are shown in Fig. 1. Although an initial higher response rate was observed in patients treated with intensified cytarabine, the response was not maintained in the majority of patients developing such a response. Fifteen of 27 responding patients lost their response including two patients with blast crisis, six patients with accelerated disease, and seven patients showed complete loss of previously established cytogenetic response. Focusing on complete cytogenetic responses: six patients in the intensified cytarabine arm obtained a complete cytogenetic response (Table 2), which lasted for a median of 5 months (range: 1-10). In the standard-arm only one patient achieved a complete cytogenetic response, which has persisted for 5 years.

Table 2. *Cumulative incidence of hematologic and cytogenetic responses in the first year of treatment*

Type of response	High-dose cytarabine	Low-dose cytarabine	P-value
	(N=32)	(N=28)	
Hematologic response			ns
Complete	24 (75%)	21 (75%)	
Partial	7 (22%)	3 (11%)	
Failure	1 (3%)	4 (14%)	
Cytogenetic response			.02
Complete	6 (19%)	1 (4%)	.11
Partial	10 (31%)	6 (21%)	
Minor	11 (34%)	9 (32%)	
Absent	5 (16%)	12 (43%)	

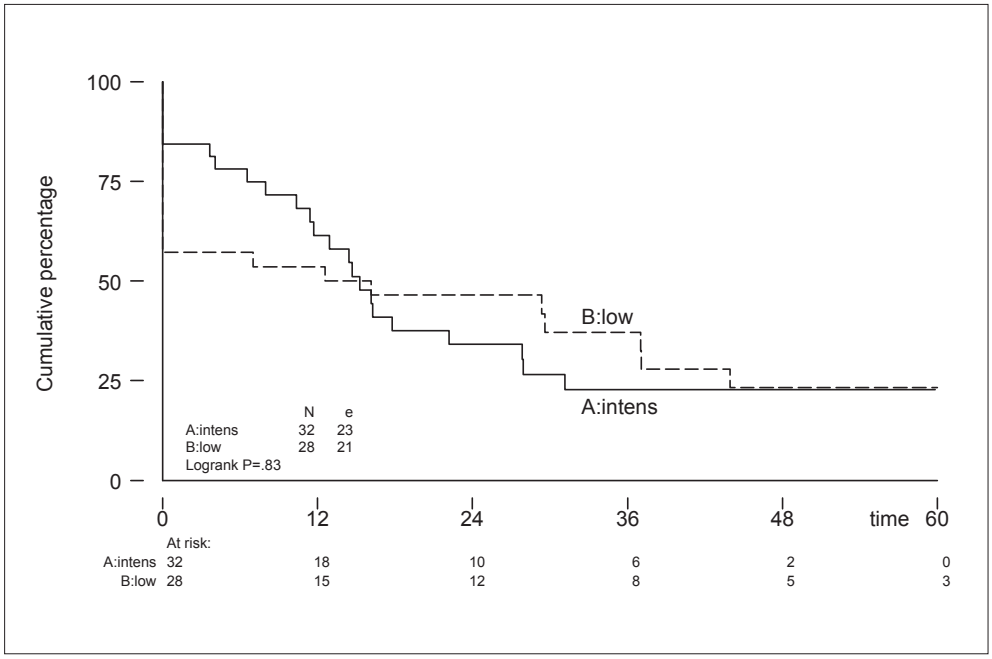

Figure 1. Event-free survival from randomization

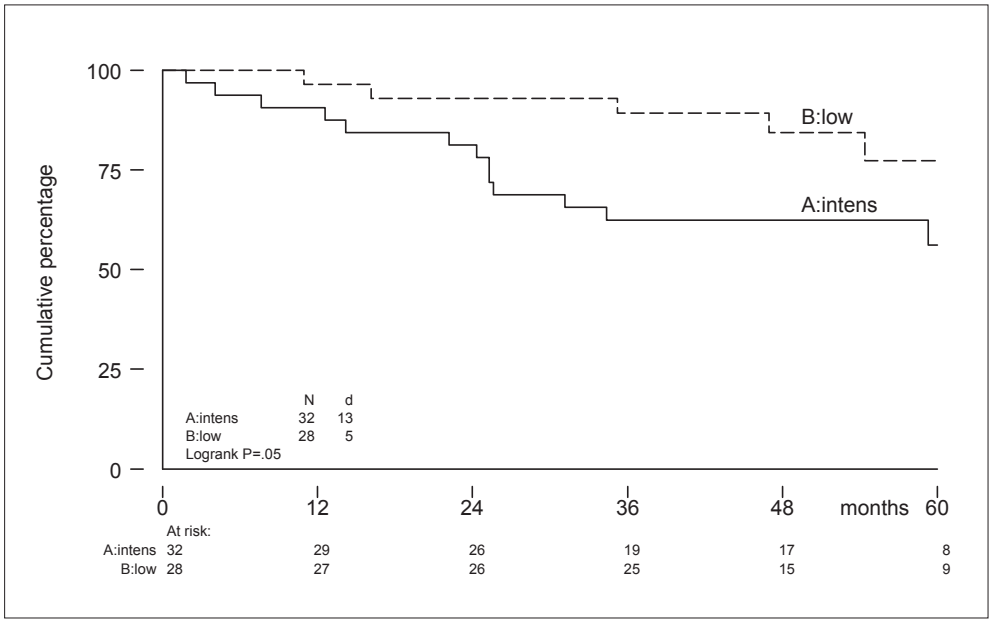

Figure 2. *Overall survival from randomization*

The median follow-up of the 42 randomized patients who are still alive, is 58 months (rang: 34-76). Eighteen randomized patients have died, 13 in arm A and five in arm B (Table 3). Causes of death in Group A included one patient with accelerated disease, six patients with blast crisis, three patients due to treatment related mortality (TRM) and three patients due to TRM secondary to a matched unrelated donor (MUD) allo-SCT. In contrast, five patients died in arm B, including two patients following accelerated disease, two patients following blast crisis and one patient as a result of an unrelated illness. Consequently, at 5-years the estimated survival rates were 56% (95% CI, 35-73%) in group A and 77% (95% CI, 52-90%) in group B (*P*=.05). Results are shown in Fig. 2.

Table 3. *Causes of death according to treatment arm*

Cause	High-dose Cytarabine	Low-dose Cytarabine	Allogeneic transplantation
	(N=13)	(N=5)	(N=21)
Accelerated disease	1	2	3
Blast crisis	6	2	1
Treatment related mortality (TRM)	3	-	15
TRM after matched unrelated donor transplantation	3	-	-
Unrelated illness	-	1	2

Discontinuation of treatment

Protocol treatment was received for a median time of 15 months in group A, versus a median time of 19 months in group B. In both arms, one patient was still on protocol treatment. Reasons for going off-protocol in group A included loss of hematologic or cytogenetic response in six patients, accelerated disease or blast crisis in seven patients. In addition, 18 patients changed to off-protocol treatment due to side effects of IFN-α or preference for imatinib. Reasons for going off-protocol treatment in group B included loss of hematologic or cytogenetic response in four patients and accelerated disease or blast crisis in five patients. Side effects of IFN-α or a preference for imatinib were causes for off-protocol treatment in another 18 patients. Ultimately, 22 patients in group A and 22 patients in group B switched to imatinib therapy. Matched unrelated donor allo-SCT was performed in eight patients in group A and in six patients in group B. These transplants were performed because of absence of hematologic response in one patient, loss of cytogenetic response in four patients and accelerated disease or blast crisis in nine patients.

Table 4. *Patient and donor and graft characteristics of allo-SCT recipients*

Parameter	No.
Patient	
CMV+	20
CMV-	27
Unknown	2
Conditioning regimen	
Cyclophosphamide/TBI	43
Busulphan/Cyclophosphamide	6
Donor	
age (yr) median, range	42 (13-64)
sex	
female	25
male	24
CMV+	21
CMV-	22
unknown	6
Donor relation to patient	
HLA-identical sib	47
identical twin	2
Source of stem cells	
bone marrow	33
peripheral blood	16
Nucleated cells infused	
$CD34^+$ 10^9/l (median, range)	2.4 (0.6-8.5)
T-cell depletion	
no	12
yes	37
Immunosuppression	
Ciclosporine	33
Ciclosporine and MTX	6
other	3
no medication	7

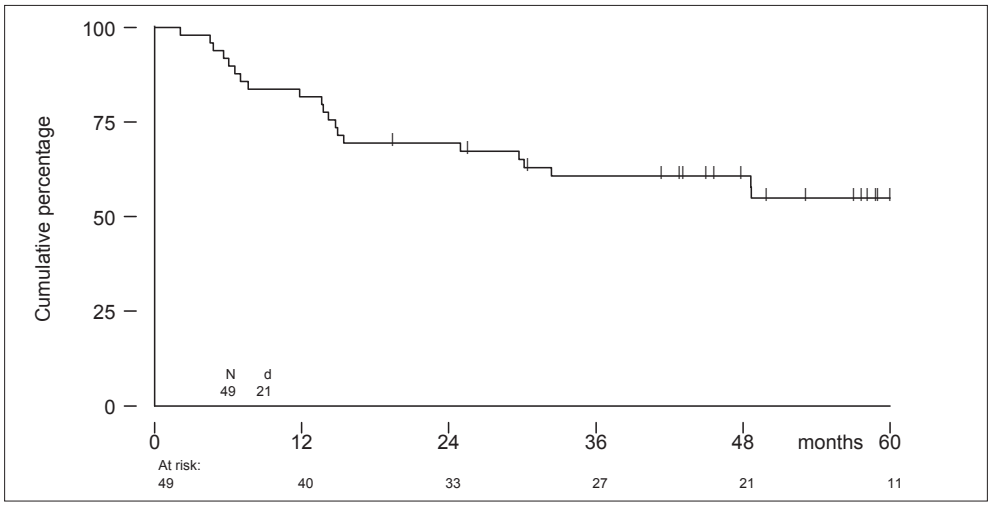

Figure 3. *Overall survival from allogeneic stem cell transplantation*

Toxicity and side effects

In group A 49 episodes of grade II, 11 episodes of grade III and nine episodes of grade IV toxicities were reported in 58 cycles. Grade IV toxicities included: liver function abnormalities in two patients, diarrhea in one, drug related fever in one, cutaneous lesions in one, cardiac arrhythmia in one, dyspnoe in two and ARDS in one. Two toxic deaths were reported in this group. In group B 35 episodes of grade II, nine episodes of grade III and no grade IV toxicities were reported in 74 periods of 6 months treatment. More grade II-IV infections were observed in group A: 42 versus 7 episodes in group B.

Allogeneic stem cell transplantation

Conditioning therapy for allo-SCT was myeloablative in all patients and consisted of total-body irradiation (TBI) in combination with cyclophosphamide in 43 patients and chemotherapy only (busulfan and cyclophosphamide) in six patients. Table 4 shows details with respect to conditioning therapy, graft characteristics and immunosuppressive regimen applied. Sixteen patients developed acute grade II-IV graft versus host disease (GVHD), chronic GVHD was observed in 25 patients, in 10 limited, and extended in 15. After a median follow-up of 58 months (range: 19 - 77), 21 patients had died. Causes of death included TRM in 15 patients, progression of CML in four patients: accelerated disease in three and blastic crisis in one, and intercurrent illness in two patients. Donor lymphocyte infusions (DLI) were given to 25 of the 49 who received an allo-SCT. In four patients it was given prophylactically, in five patient for molecular residual disease, in 14 patients as a treatment for cytogenetic relapse and in two patients following accelerated disease or blast crisis. Thirteen patients became PCR negative again after DLI. Recipients of allo-SCT showed a 5-years estimated survival of 55% (95% CI, 39-68%). Results are shown in Fig. 3.

Discussion

In this prospective randomized study, started before the introduction of imatinib, we compared intensified cytarabine followed by IFN-α maintenance with low-dose cytarabine combined with IFN-α. The design of the study was based on earlier experience by us and others[4,7-9] and on the assumption that cytarabine may exert a dose dependent effect as can be observed in AML.[5,6] In addition, as previous studies evaluating more intensive chemotherapy showed relatively high rates of cytogenetic relapse, we added IFN-α maintenance following two courses of chemotherapy in order to prevent relapse. The cytogenetic response rate proved superior in the intensified arm (P=.02), the majority of patients lost their initial cytogenetic response emphasizing that IFN-α maintenance proved to be insufficient to preserve the initial cytogenetic response. As a result, EFS was comparable among the two treatment arms.

The lack of a sustained beneficial effect of intensified cytarabine followed by IFN-α maintenance may be explained in several ways. Cytarabine acts almost exclusively on proliferating cells. Apart from a large pool of proliferating malignant mature and more immature myeloid cells, CML is also characterized by the presence of quiescent non-cycling Ph+ progenitors,[11-14] which may not have been affected sufficiently by cytarabine. As earlier shown by Holyoake et al., these progenitors may have a proliferative advantage over their quiescent normal counterparts and may, hereby, be responsible for gradually resuming malignant hematopoiesis after recovery from cytarabine induced cytopenia.[12] Interferon-alfa maintenance did not prevent the regrowth of malignant hematopoiesis in most patients with an initial cytogenetic response. In view of the relatively low rates of complete cytogenetic responses to IFN-α,[3,15] also IFN-α may have been unable to eliminate malignant progenitor cells that were responsible for the recapitulation of malignant hematopoiesis in our patients. The relatively low-dose of IFN-α administered after intensified cytarabine may have contributed to that effect. However, reports in literature do not support a dose response effect for IFN-α. A meta-analysis of three prospective controlled trials comparing low-dose and high-dose IFN-α showed no benefit for high-dose therapy.[16]

What has been accomplished with intensive therapy so far? Kantarjian et al. treated 32 patients with intensive chemotherapy consisting of daunorubicin, cytarabine, vincristine and prednisone followed by IFN-α maintenance.[9] Overall, 60% of patients showed a major cytogenetic response, but only eight patients (25%) had a sustained cytogenetic response. No survival benefit was observed compared with a matched historical control group. Improved survival by intensive chemotherapy was suggested by Giles et al. in patients who did not benefit from IFN-α.[10] The projected 10-year survival was 50% for the study group versus 35% for patients who received an IFN-α based regimen at the MD Anderson. Alternatively, chemotherapy may be further intensified and supported by autologous transplantation. McGlave et al. summarized results of 8 transplant centres.[17] One hundred and forty-two patients in first chronic phase received an autologous transplantation. The 3-year survival was approximately 60% with some indication of a plateau after 3.6 years in the survival curve. Simonsson et al reported the Swedish results with autologous transplantation.[18] Initial treatment included hydroxyurea and IFN-α and one to three courses of intensive chemotherapy. The actuarial 6-year survival from diagnosis of all 160 registered patients was 68%, including 30 patients having received an autograft and 59 patients who were allografted. The survival of 581 patients retrieved from the EBMT registry who received an autologous transplantation between 1983 and 1998 were reported by Olavarria et al.[19] The median survival was 8 years from SCT without indication of a plateau in survival curve. Patients with a major cytogenetic response 1 – 2 years post SCT and also patients who received IFN-α post SCT did significantly better. Although none of these studies show definite proof of a survival advantage with intensive therapy, a subgroup of yet to be defined patients could potentially benefit from more intensive chemotherapy.

Is there still a role for chemotherapy in the current imatinib era? In the IRIS trial, the estimated rate of major cytogenetic response and complete cytogenetic response rates were 87% and 76% in the imatinib group, respectively, compared with 35% and 15% in the group given IFN-α plus cytarabine at 18 months.[20] Less then 5% of patients achieved a complete molecular response. In patients with a major molecular response within 12 months, estimated progression free survival at 24 months was 100% compared with 95% for patients in complete cytogenetic response without a 3-log reduction of BCR-ABL and 85% for patients who did not have a complete cytogenetic remission.[21] As a result, the current goal of treatment in first chronic phase CML patients is to aim for a major molecular response.[21,22] Resistance, however, is a major concern. Different mechanisms of resistance against imatinib have been reported, including, mutations of the BCR-ABL kinase domain, gene amplification or overexpression of BCR-ABL, clonal evolution, overexpression of the multidrug resistance P-glycoprotein and inactivation by α-1 acid glycoprotein.[23-27] Currently, combination therapies are being developed to prevent resistance or to treat patients that have acquired resistance.[28] Apart from the combination of imatinib with alternative signal transduction inhibitors, also the combination with conventional chemotherapy is being explored. With respect to the combination of imatinib and cytarabine, two groups have reported initial results. The French CML group performed a phase II trial in 30 patients.[29] A complete cytogenetic response was obtained in 70% of the patients at 12 months. We performed a phase I/II study exploring the feasibility and efficacy of escalating doses of imatinib and two different dosages of cytarabine.[30] The probability of reaching a complete cytogenetic response was 67%, a major molecular response was 51% and a complete molecular response was 28% at 18 months of treatment. While these initial molecular results seem promising, longer follow-up is needed to evaluate whether cytarabine significantly adds to the efficacy of imatinib and may prevent resistance.

In conclusion, high-dose cytarabine based chemotherapy followed by IFN-α maintenance was not better as compared to low-dose cytarabine in combination with IFN-α in early diagnosed CML patients. As imatinib may reverse the relative insensitivity to chemotherapeutic agents,[31,32] the role of chemotherapy now deserves to be defined in particular in combinations with imatinib, with the purpose of preventing and treating tyrosine kinase inhibitor resistant CML.

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DOSE-FINDING STUDY OF IMATINIB IN COMBINATION WITH INTRAVENOUS CYTARABINE: FEASIBILITY IN NEWLY DIAGNOSED PATIENTS WITH CHRONIC MYELOID LEUKEMIA.

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Abstract

The HOVON cooperative study group performed a feasibility study of escalated imatinib and intravenous cytarabine in 165 patients with early chronic-phase chronic myeloid leukemia (CML). Patients received 2 cycles of intravenous cytarabine (200 mg/m^2 or 1000 mg/m^2 days 1-7) in conjunction with imatinib (200 mg, 400 mg, 600 mg or 800 mg), according to predefined, successive dose levels. All dose levels proved feasible. Seven dose-limiting toxicities (DLTs) were observed in 302 cycles of chemotherapy, which were caused by streptococcal bacteremia in 5 cases. Intermediate-dose cytarabine (1000 mg/m^2) prolonged time to neutrophil recovery and platelet recovery compared with a standard-dose (200 mg/m^2). High-dose imatinib (600 mg or 800 mg) extended the time to platelet recovery as compared with a standard-dose (400 mg). More infectious complications common toxicity criteria (CTC) grade 3 or 4 were observed after intermediate-dose cytarabine compared with a standard-dose of cytarabine. Early response data after combination therapy included a complete cytogenetic response in 48% and a major molecular response in 30% of patients, which increased to 46% major molecular responses at 1 year, including 13% complete molecular responses. We conclude that combination therapy of escalating dosages of imatinib and cytarabine is feasible. This study was registered at *www.kankerbestrijding.nl* as no. CKTO-2001-03.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by a reciprocal translocation between the long arms of chromosomes 9 and 22, known as the Philadelphia (Ph) translocation.[1,2] The molecular consequence of this translocation is the generation of a bcr-abl fusion gene, which encodes for a chimeric protein with constitutive tyrosine-kinase activity sufficient for leukemogenesis in mice.[3] Imatinib is a relatively specific inhibitor of the BCR-ABL tyrosine kinase and acts by stabilizing the inactive non-ATP-binding conformation of BCR-ABL. In the International Randomized Study of Interferon and STI571 (IRIS) a complete hematologic response was obtained in 98% of the patients and a complete cytogenetic response in 87% of the patients with newly diagnosed CML after a median follow-up of 60 months.[4,5] Imatinib has become the drug of choice as first-line therapy in the treatment of CML. However, the development of resistance is of concern. The estimated rate of event-free survival in the IRIS study was 83% at 60 months, while an estimated 7% of all patients progressed to accelerated phase or blast crisis. Patients who did not obtain a complete hematologic response at 3 months, a minor cytogenetic response at 6 months, a major cytogenetic response at 12 months or a complete cytogenetic response at 18 months were at increased risk of relapse.[5,6]

The question has arisen whether it is possible to increase the molecular response rate and prevent resistance by combination therapy. In vitro studies have shown synergistic action between imatinib and various drugs, including cytarabine.[7,8] Cytarabine is a very active drug and probably the most potent drug in acute myeloid leukemia.[9,10] Low-dose cytarabine in combination with interferon alfa (IFN-α) was considered standard treatment before the introduction of imatinib,[11] and higher dosages of cytarabine were associated with better response rates.[12] The synergistic activity observed in vitro by combining imatinib and cytarabine was especially observed when both drugs were applied in increasing concentrations.[7,8] A clear dose-response relationship has been established for imatinib monotherapy, and an increased rate of molecular remission was suggested in patients treated with 800 mg of imatinib.[13] These findings have evoked the question whether the combination of cytarabine and imatinib may improve response and prevent resistance. In view of the dose-dependent effects of both drugs, we explored the feasibility of the combination of imatinib and cytarabine using escalating dosages in successive dose levels.

Methods

Patients with newly diagnosed CML in first chronic-phase were eligible if they were between 18 and 65 years of age and registered within 6 months of diagnosis. Other eligibility criteria included the presence of the Philadelphia chromosome or BCR-ABL rearrangement and WHO performance status status 2 or less. Previous treatment for CML was not allowed with the exception of hydroxyurea. Patients with hepatic dysfunction, renal insufficiency, severe cardiac, pulmonary or neurologic disease, active uncontrolled infections, human immunodeficiency virus infection, and malignancies during the past 5 years with the exception of basal carcinoma of the skin or stage 0 cervical carcinoma, and pregnant or lactating women were not eligible. Patients with a human leukocyte antigen (HLA)-matched sibling donor who were scheduled to receive an allogeneic transplantation upfront were also ineligible. The study was approved by the ethics committees of the participating institutions, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Study design and treatment

Treatment with imatinib was started, after discontinuation of hydroxyurea, at a dose of 400 mg once daily and continued for 2 to 3 weeks. This prephase of imatinib monotherapy was designed to avoid cumulative toxicity of hydroxyurea and cytarabine. Thereafter, patients were hospitalized to receive the first of 2 cycles of intravenous cytarabine in conjunction with oral imatinib. Imatinib was given once daily at a dose of either 200 mg, 400 mg, 600 mg or 800 mg in combination with standard-dose cytarabine (200 mg/m^2) in a 2 hours infusion or intermediate-dose cytarabine (1000 mg/m^2) in a 3 hours infusion days 1 to 7, according to the assigned dose level I-V (Figure 1). Patients who received standard-dose cytarabine were discharged after chemotherapy and readmitted when they became neutropenic. Patients who received intermediate-dose cytarabine were hospitalized until hematologic recovery. Prophylaxis for prevention of Gram-negative bacterial and fungal infections was mandatory until resolution of neutropenia and penicillin prophylaxis was given at days 8-20 of intermediate-dose cytarabine only.

Initially, 5 patients were entered in the lowest dose level (cytarabine 200 mg/m^2 and imatinib 200 mg). The study was thereafter temporarily put on hold until these patients could be evaluated for dose-limiting toxicity (DLT). Patients who went off protocol before completion of cycle I for reasons not related to DLT were replaced. Depending on the number of patients with a DLT or patients who died of treatment related mortality (TRM) during or after cycle I, inclusion of patients continued in the same or in the next higher dose level, according to the decision rules specified in Table 1. In short, a subsequent dose level was open for inclusion when the criteria of acceptable toxicity and safety had been met (i.e. when ≤ 5% TRM and ≤ 20% DLT [including TRM] had been observed in that dose level).

In addition, inclusion in the next dose level was put on hold if evaluation of the preceding dose level was not completed, while inclusion and extension of the preceding dose level was allowed. Dose levels IIIA and IIIB were opened simultaneously after the previous dose level had met the criteria of acceptable toxicity and safety and afterwards, when both dose levels were proved feasible, dose levels IVA and IVB were also opened simultaneously. Dose level V was started after IVA and IVB had met the criteria of acceptable toxicity and safety.

The second cycle was given after full hematologic recovery (platelets > 100 x 10^9/l and white blood cell count (WBC) > 2.0 x 10^9/l). Cycle II was preferably not given before day 28 and not later than day 42 from the start of cycle I. No dose modifications were made for cytarabine during combination therapy. Imatinib was continued after chemotherapy during the phase of neutropenia and thrombocytopenia, but withheld in case of CTC grade 4 stomatitis if this persisted for more than a week. Imatinib was also withheld in case of CTC grade 3 or 4 liver toxicity and any other CTC grade 4 toxicity except for hematologic toxicity, nausea, and vomiting. When toxicity had resolved (< grade 2), therapy was resumed at the same dose. After the second cycle of combination therapy, imatinib maintenance therapy was given at the same dose as given during cytarabine treatment. Dose adjustments were made for non-hematologic toxicity CTC grade 2 or higher and for hematologic toxicity CTC grade 4 or higher during maintenance therapy with imatinib. Imatinib maintenance therapy was continued until progression. Other reasons for going off protocol treatment were excessive toxicity, including toxic death, intolerance of treatment, intercurrent death, no compliance of the patient, major protocol violation or proceeding to allogeneic stem cell transplantation.

Table 1. *Number of patients per dose level and decision rules*

No. of evaluable patients	No. of patients with			Action
	DLT	And/Or	TRM	
5	0 - 1	And	0	Go to dose level N+1 with entry of 5 patients
5	5	Or	≥ 2	STOP; dose level N not feasible [1)]
5	2 - 4	Or	1	Enter 5 more patients at dose level N
10	0 - 2	And	0	Go to dose level N+1 with entry of 5 patients
10	≥ 5	Or	≥ 2	STOP; dose level N not feasible [1)]
10	3 - 4	Or	1	Enter 10 more patients at dose level N
15	0 - 3	And	0	Go to dose level N+1 with entry of 5 patients
15	≥ 5	Or	≥ 2	STOP; dose level N not feasible [1)]
15	4	Or	1	Enter 5 more patients at dose level N
20	0 - 4	And	0 - 1	Go to dose level N+1 with entry of 5 patients
20	≥ 5	Or	≥ 2	STOP; dose level N not feasible [1)]

N indicates current dose level

[1)] Enter a total of 20 patients at dose level N-1, continue entry according to the decision rules for dose level N-1.

Definition of end points

Dose-limiting toxicities were defined as toxicities with onset within 42 days after the start of cycle I or II of the following type and grade: CTC grade 4 mucosal, hepatic enzyme or bilirubin toxicity lasting more than 2 weeks. Any other CTC grade 4 non-hematologic toxicity and any TRM occurring after start of cycle I was also defined as DLT. Treatment related mortality was defined as death related to the combination treatment of imatinib and cytarabine, as judged by the responsible local investigator. Feasibility was defined by TRM occurring in 5% or less of patients and DLT (including TRM) occurring in ≤ 20% of patients in a dose level.

Time to hematologic recovery (neutrophil count [ANC] > 0.5 x 10^9/l , and platelet count > 50 x 10^9/l, was calculated from the first day below the threshold to recovery. Criteria for a complete hematologic response were normalization of the white blood cell count to less than 10 x 10^9/l with no immature forms with the exception of 2% or less myelocytes and metamyelocytes, a platelet count < 450 x 10^9/l and disappearance of all clinical symptoms and signs of disease including palpable splenomegaly. A partial hematologic response was defined as not fulfilling all the criteria for complete hematologic remission and a WBC ≤ 20 x 10^9/l. Failure was defined as WBC > 20 x 10^9/l, or progression to accelerated phase or blast crisis. Cytogenetic response was classified as absent (100% Ph chromosome positive metaphases), minor (35-99% Ph chromosome-positive metaphases), partial (1-34% Ph chromosome positive metaphases), or complete (elimination of Ph chromosome positive metaphases), as determined in the local cytogenetic referral center, on the basis of G-, R-, or Q-banding in at least 20 metaphase cells per sample. Cytogenetic analysis of peripheral blood was acceptable only at diagnosis. Fluorescent in situ hybridization (FISH) analysis on metaphase or interphase cells with specific BCR-ABL probesets was performed for patients with a cryptic Ph at diagnosis and follow-up and, in addition, during follow-up when cytogenetic analysis failed.

Molecular response was defined as complete (≥ 4.5-log reduction of BCR-ABL mRNA detectable by real-time quantitative RT-PCR), major (≥ 3-log reduction of BCR-ABL mRNA), partial (≥ 1- and < 3-log reduction of BCR-ABL mRNA), or absent (< 1-log reduction of BCR-ABL mRNA). Molecular response was centrally assessed in Rotterdam using real-time quantitative PCR (RQ-PCR). Bone marrow samples for PCR analysis were required at diagnosis; immediately following combination therapy; and at regular (6 months) intervals thereafter. Patients with molecular responses were monitored by PCR of peripheral blood at 3-6 months intervals, and also by PCR of bone marrow once yearly.[14] First, total RNA was extracted from bone marrow or peripheral blood using RNABee (Campro Scientific, Veenendaal, The Netherlands). Afterwards, cDNA was synthesized from 1 μg of RNA using random hexamer priming, essentially as described.[15] cDNA prepared from 25 ng of RNA was used for all PCR amplifications. RQ-PCR amplification was performed with the ABI

PRISM 7700 or 7500 Sequence Detector (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), using 25 µL mix containing 125 µM deoxyribonucleoside triphosphates (dNTPs; Amersham Pharmacia Biotech, Roosendaal, The Netherlands) 7.5 pmol forward and 7.5 pmol reversed primer (BCR-ABL: T.BA FOR 5'- CCGCTGACCATCAATAAGGAA - 3' and T.BA REV 5'-TCAGACCCTGAGGCTCAAAGTC -3'; PBGD: PBGD FOR 5'- GGCAATGCGGCTGCAA -3' and PBGD REV 5'- GGTACCCACGCGAATCAC -3'); 1 mM $MgCl_2$; 4 pmol probe for ABL (5'-AAGCCCTTCAGCGGCCAGTAGCA - 3') and 5 pmol probe for PBGD (5'- CATCTTTGGGCTGTTTTCTTCCGCC - 3'), both labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3' end with the quencher dye molecule TAMRA (6-carboxy-tetramethylrhodamine) (Eurogentec, Maastricht, The Netherlands), 1 x buffer A; 1.25 U AmpliTaq Gold with the PBGD; and 2.5 U AmpliTaq Gold with the BCR-ABL amplification (Applied Biosystems). The thermal cycling conditions for BCR-ABL and PBGD included 10 minutes at 95˚C followed by 45 cycles of denaturation for 15 seconds at 95˚C, annealing at 58˚C for 30 seconds, and extension at 60˚C for 30 seconds.

The relative expression levels of BCR-ABL were quantified using a standard curve of serial dilutions of the calibrator K562 and were normalized using the endogenous reference PBGD. The level of BCR-ABL expression of the undiluted K562 is representative for a CML patient in chronic phase at diagnosis. All RQ-PCR amplifications reached a sensitivity of at least 10^{-4} (K562/HL60) in duplicate and an efficiency of at least 93%.

Assessment of toxic effects and response

Complete blood counts were obtained at least every other day and biochemical analysis at least twice weekly during combination therapy. Bone marrow assessment was done after cycles I and II, at 6 months, and there after at least every 6 months. Patients were evaluated for cytogenetic response after cycle II, at 6 months, at 12 months and once a year thereafter. Molecular analysis was done at baseline, after cycles I and II, at 6 months and at least every 6 months thereafter. Safety assessments included an evaluation of adverse events, hematologic assessment, biochemical testing, urinalysis, and physical examination. Electrocardiography and chest X-ray were done at baseline and if clinically indicated thereafter.

Statistical considerations

The primary objective of this study was to determine the feasibility of the combination of imatinib and cytarabine in a dose-escalation study of consecutive cohorts. Secondary end points were the rate and duration of complete hematologic response, the rate and duration of complete cytogenetic response, and the rate and duration of complete molecular response. Progression was defined by the first occurrence of any of the following events: the development of accelerated phase or blast crisis, complete loss of hematologic response, loss of major cytogenetic response (defined as an increase in Ph-positive cells in metaphase by at

least 30 percentage points on two cytogenetic analyses performed at least one month apart) or an increasing white-cell count (defined as doubling of the count to > 20 x 10^9/l on two occasions at least one month apart in a patient who had never had a complete hematologic response despite receiving maximally tolerated doses of therapy). Other secondary end points included side effects and infections, hematologic recovery, time to mutation of the ABL-kinase domain, progression-free survival, and overall survival. Side effects; infections, hematologic recovery; and hematologic, cytogenetic and molecular response data after one or two cycles of combination therapy and after one year are shown in this report. Actuarial response rates at one year were calculated using competing risk analysis; patients who went off protocol treatment before the specific response had occurred were considered as competing risks. Other secondary end points will be presented separately, after having obtained sufficient follow-up. Special attention was given to non-hematologic toxicity, hematologic toxicity and infectious complications during combination therapy. Side effects and infections were scored according to the National Cancer Institute (NCI) common toxicity criteria (CTC) version 2.0. Hematologic recovery was estimated by the Kaplan-Meier method. Kaplan-Meier curves were generated to illustrate differences in recovery between standard- and intermediate-dose cytarabine, as well as between low/standard-dose (200 and 400 mg) and high-dose (600 and 800 mg) imatinib and were compared using the log-rank test. All reported *P*-values are 2-sided, and a significance level $\alpha = .05$ was used.

Results

From August 2001 to November 2005, 165 patients entered the study. Five patients were assigned to dose level I, 30 patients, to dose level II, 21 patients to dose level IIIA, 16 patients to dose level IIIB, 52 patients to dose level IVA, 21 patients to dose level IVB and 20 patients to dose level V (Figure 1). Three patients were excluded from analysis: one was not considered because blast crisis was diagnosed shortly after registration and before start of imatinib (dose level IVB), and two other patients were not evaluable because they refused combination therapy (dose levels II and IIIB). The analysis reported here describes 162 patients and includes the feasibility and response of the pre-phase and two cycles of chemotherapy in combination with imatinib.

Patient baseline characteristics are presented according to the dose of cytarabine received (200 mg/ m^2 versus 1000 mg/m^2) and are summarized in Table 2. All but two patients received a pre-phase of imatinib monotherapy 400 mg once daily for three weeks (median 21 days, range 5 – 84 days). All 162 patients received at least one cycle of combination therapy. Five patients in dose level I, 28 patients in dose level II, 18 patients in dose level IIIA, 11 patients in dose level IIIB, 45 patients in dose level IVA, 17 patients in dose level IVB and 16 patients in dose level V received both scheduled courses of combination therapy. The remaining 22

patients did not receive a second course of combination therapy because of non-hematologic toxicity in 8 patients, insufficient hematologic recovery in 10 patients, and refusal in four patients. The dose of cytarabine was given as scheduled, except for one patient who received a mitigated dose because of central nervous system toxicity. A reduction of the scheduled dose of imatinib was performed in 31 patients during the first course and in 23 patients during the second course according to predefined dose-adaptation rules. One hundred and fifty-seven patients started with imatinib maintenance therapy, including 19 patients who had received only one cycle of combination therapy. Five patients did not start with imatinib maintenance because of toxicity in two patients, progression in one patient, and intercurrent death in two patients.

Table 2. *Baseline characteristics of the patients, by dose of cytarabine*

Characteristic	200 mg/m² cytarabine (dose levels I, II, IIIA, IVA)	1000 mg/m² cytarabine (dose levels IIIB, IVB, V)
	(N=107)	(N=55)
Age at diagnosis, y		
median	48	46
range	20-65	19-62
Sex, no. (%)		
male	64 (60%)	31 (56%)
female	43 (40%)	24 (44%)
Spleen size, cm below midleft costal margin		
median	3	1
range	0-27	0-30
Platelet count, x 10^9/l		
median	412	357
range	152-1908	92-1584
Blasts in peripheral blood (%)		
median	1	1
range	0-12	0-16
Sokal risk group, no. (%)		
low < 0.8	30 (28%)	28 (51%)
intermediate 0.8-1.2	40 (37%)	11 (20%)
high > 1.2	31 (29%)	12 (22%)
unknown	6 (6%)	4 (7%)
Dose imatinib, no. (%)		
200 mg	5 (5%)	-
400 mg	29 (27%)	15 (27%)
600 mg	21 (20%)	20 (36%)
800 mg	52 (49%)	20 (36%)

Percentages may not sum up to 100% due to rounding

Dose-limiting toxicities and treatment related mortality

All dose levels met predefined feasibility criteria. Dose-limiting toxicities were reported in seven patients. Streptococcal infections associated with DLTs were diagnosed in five patients. Toxicities in these patients were considered a consequence of streptococcal bacteremia, including two patients with cerebral abscesses (Table 3). Two out of these five patients succumbed following these septic episodes. Four of the five infectious DLTs occurred after intermediate-dose cytarabine. In three patients these DLTs occurred after penicillin prophylaxis was stopped according to protocol. One patient experienced streptococcal septicemia during levofloxacine prophylaxis and one other patient had not received prophylaxis according to protocol. Two other DLTs included myalgia CTC grade 4 and an anaphylactic reaction following platelet transfusion. All DLTs were observed during the first cycle.

Side effects and infections

The CTC grade 3 and 4 non-hematologic and non-infectious toxicities are listed in Table 4. The incidence of these toxicities was comparable between patients receiving either the standard- or intermediate-dosage cytarabine. Most patients receiving combination therapy developed hematologic toxicity CTC grade 4. Infectious complications CTC grade 3 or 4 were diagnosed in 48 patients (87%) after intermediate-dose cytarabine as compared to 46 patients (43%) after standard-dose cytarabine (P<.001) and are listed in table 5. Most infectious complications occurred after the first cycle of cytarabine (Table 5). The dose of imatinib did not influence the incidence of infectious complications (data not shown).

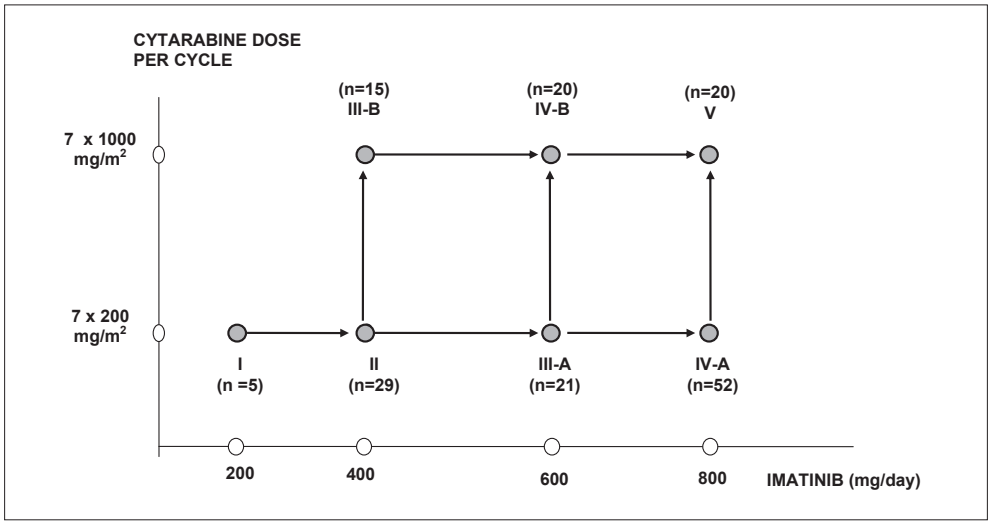

Figure 1. *Successive dose levels, by dose cytarabine and imatinib*

Table 3. *Dose-limiting toxicity and treatment related mortality*

Dose level	Cycle	Specify	Treatment related mortality
			Yes / No
III-A	I	Streptococcus mitis sepsis, acute respiratory distress syndrome, hypotension and cerebral abscesses	Yes
III-B	I	Streptococcus species bacteriaemia with transient cerebral edema	No
IV-A	I	Anaphylactic reaction on platelet transfusion	No
IV-A	I	Myalgia CTC grade 4	No
V	I	Streptococcus viridans sepsis, cerebral abscesses and Aspergillus fumigatus pneumonia	Yes
V	I	Streptococcus mitis sepsis and pneumonia, liver toxicity CTC grade 4 and renal failure (acute tubular necrosis)	No
V	I	Streptococcus oralis bacteriaemia and myalgia CTC grade 4	No

CTC indicates common toxicity criteria

Table 4. *Number of patients with non-hematologic adverse events CTC grade 3 or 4 (percentages), by daily dose cytarabine and by cycle number*

Adverse event	cytarabine 200 mg/m² (dose levels I, II, IIIA, IVA)		cytarabine 1000 mg/m² (dose levels IIIB, IVB, V)	
	Cycle 1 (n=107)	Cycle 2 (n=96)	Cycle 1 (n=55)	Cycle 2 (n=44)
Any	23 (21%)	14 (15%)	14 (25%)	11 (25%)
Hemorrhage	8 (7%)	5 (5%)	3 (5%)	1 (2%)
Neurology	4 (4%)	1 (1%)	3 (5%)	1 (2%)
Hepatic	2 (2%)	2 (2%)	3 (5%)	1 (2%)
Pain	8 (7%)	1 (1%)	3 (5%)	2 (5%)
Cardiovascular function	1 (1%)	1 (1%)	2 (4%)	2 (5%)
Constitutional symptoms	1 (1%)	1 (1%)	1 (2%)	3 (7%)
Dermatology/skin	-	4 (4%)	2 (4%)	-
Gastrointestinal	2 (2%)	1 (1%)	3 (5%)	4 (9%)
Metabolic	1 (1%)	-	1 (2%)	-
Pulmonary	1 (1%)	-	1 (2%)	1 (2%)
Allergy/immunology	2 (2%)	-	1 (2%)	-
Genitourinary and renal	-	-	1 (2%)	-

Table 5. *Number of patients with infectious episodes CTC grade 3 or 4 (percentages), by daily dose cytarabine and by cycle number*

Infections	cytarabine 200 mg/m² (dose levels I, II, IIIA, IVA)		cytarabine 1000 mg/m² (dose levels IIIB, IVB, V)	
	Cycle 1 (n=107)	Cycle 2 (n=96)	Cycle 1 (n=55)	Cycle 2 (n=44)
Any	36 (34%)	23 (24%)	44 (80%)	24 (55%)
Fever of unknown origin	17 (16%)	9 (9%)	23 (42%)	7 (16%)
Blood				
Staphylococcus	1 (1%)	-	6 (11%)	4 (9%)
Streptococcus	2 (2%)	-	4 (7%)	2 (5%)
Pseudomonas aeruginosa	-	-	1 (2%)	1 (2%)
Other/unknown	-	1 (1%)	2 (4%)	2 (5%)
Gastrointestinal tract	4 (4%)	3 (3%)	8 (15%)	8 (18%)
Ear/nose/throat	7 (7%)	6 (6%)	4 (7%)	2 (5%)
Skin/subcutaneous	5 (5%)	3 (3%)	1 (2%)	2 (5%)
Pulmonary				
Aspergillus	-	1 (1%)	3 (5%)	1 (2%)
Streptococcus	1 (1%)	-	1 (2%)	-
Other/unknown	3 (3%)	-	4 (7%)	-
Catheter	1 (1%)	1 (1%)	2 (4%)	5 (11%)
Genitourinary tract	2 (2%)	1 (1%)	2 (4%)	2 (5%)
Other	2 (2%)	3 (3%)	-	2 (5%)

Hematologic recovery

The time to neutrophil recovery to more than 0.5 x 10^9/l was significantly longer following intermediate-dose cytarabine compared with a standard-dose cytarabine (P<.001; Figure 2). The median number of days of neutropenia ≤ 0.5 x 10^9/l was 13 days (range 0-36) following cytarabine (200 mg/m²) compared with 19 days (range 7-47) following cytarabine (1000 mg/m²) in the first cycle. Platelet recovery to > 50 x 10^9/l was also significantly more protracted following intermediate-dose cytarabine (Figure 3). Time to neutrophil and platelet recovery was also significantly prolonged after intermediate-dose cytarabine in the second cycle (data not shown). Time to platelet recovery > 50 x 10^9/l was also adversely affected by a higher dose of imatinib (600 or 800 mg). The dose of imatinib did not affect the time to neutrophil recovery. Patients received a median number of 3 platelet transfusions (range 0-16 transfusions) after standard-dose cytarabine, as compared to 5 transfusions (range 2-21 transfusions) after intermediate-dose cytarabine in the first cycle. Furthermore, patients received a median number of 3 red blood cell transfusions (range 0-13 transfusions) after standard-dose cytarabine, as compared to 4 transfusions (range 0-24 transfusions) after intermediate-dose cytarabine. Difference in transfusion requirements was largely similar after the second cycle of combination therapy.

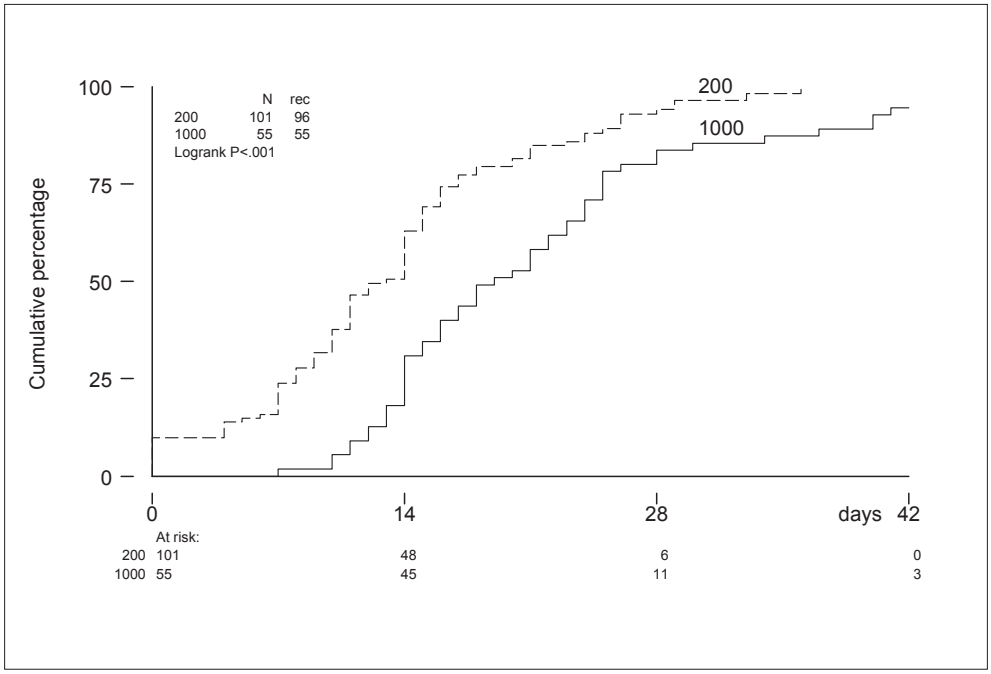

Figure 2. *Neutrophil recovery from below threshold to > 0.5 x 10⁹/l according to dose level cytarabine in cycle 1*

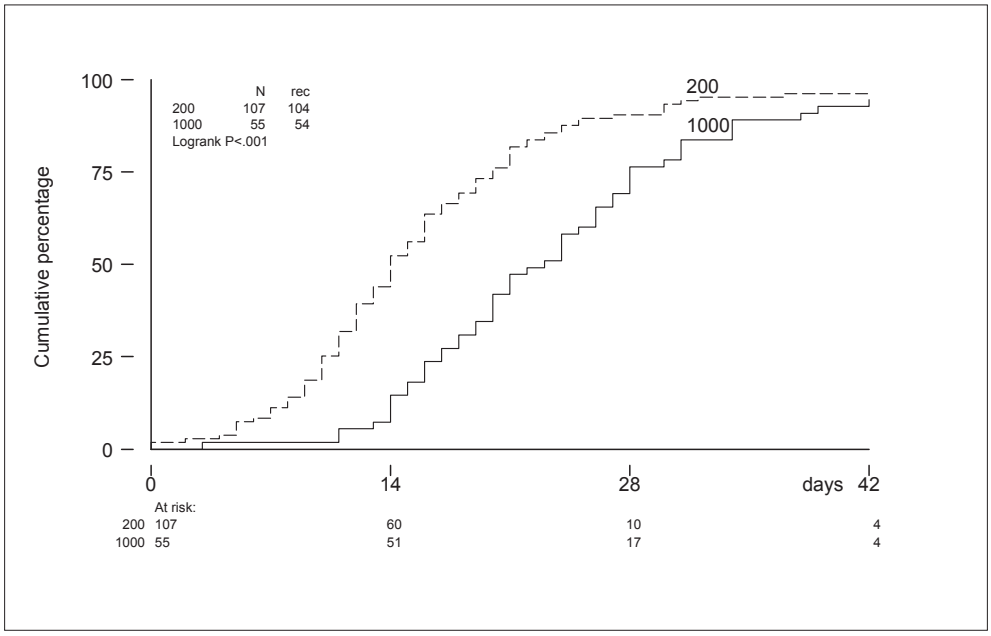

Figure 3. *Platelet recovery from below threshold to > 50 x 10⁹/l according to dose level cytarabine in cycle 1*

Hematologic, cytogenetic, and molecular responses

One hundred forty-eight patients obtained a complete hematologic response after one or two cycles of combination therapy. Eight patients obtained a partial hematologic response, one patient was unresponsive and another patient progressed to accelerated phase. The hematologic response could not be assessed appropriately in four patients due to insufficient hematologic recovery in three patients and death before evaluation in one patient. The cytogenetic response was evaluated in 133 patients after combination therapy and included a complete cytogenetic response in 64 patients (48%), a partial cytogenetic response in 36 patients (27%), a minimal cytogenetic response in 29 patients (22%), and an absent cytogenetic response in 4 patients (3%) after a median of 91 days. The molecular response was evaluated after a median of 80 days in 138 patients and included a major molecular response in 42 patients (30%, including 3 patients with a complete molecular response), a partial molecular response in 79 patients (57%), and an absent molecular response in 17 patients (12%). At 12 months, actuarial probabilities of a complete hematologic response and a complete cytogenetic response were 95% (95% confidence interval [CI]: 91%-97%) and 63% (95% CI: 55%-70%), respectively. One hundred patients achieved a complete cytogenetic response within 12 months, including 71 patients with a major molecular response. Twenty-two patients achieved a complete molecular response at that time. As a result, probabilities of major and complete molecular response were 46% (95% CI: 39%-55%) and 13% (95% CI: 9%-20%), respectively, at 1 year. Six patients progressed during the first year, including one patient who developed accelerated phase, four patients who developed blast crisis and one patient who lost his partial cytogenetic response. Four patients died, including two patients due to TRM, one patient due to progression of CML, and one patient due to death from unrelated causes.

Discussion

Given the synergistic and dose-dependent actions of imatinib and cytarabine, as was observed in in vitro studies, the HOVON-51 study was designed to investigate whether escalating doses of imatinib (200 mg, 400 mg, 600 mg, or 800 mg) combined with two cycles of intravenous cytarabine (200 mg/m² or 1000 mg/m² days 1-7) would be feasible and would induce an early molecular response in patients with first chronic-phase CML. All dose levels (I-V) proved feasible. Seven DLTs were observed among 162 patients, who had received 302 cycles of combination therapy. Five of these seven DLTs resulted from streptococcal bacteremia. More infectious complications were observed after intermediate-dose cytarabine (1000 mg/m²) compared with standard-dose cytarabine (200 mg/m²), especially after the first cycle of combination therapy. While the percentage of DLTs at dose level V was less than 20%, three DLTs were observed at that particular level, which was associated with TRM in one patient. The dose of imatinib did not affect the rate of infectious complications. Intermediate-dose cytarabine significantly prolonged the period of neutropenia and thrombocytopenia as compared to a standard-dose of cytarabine. High-dose imatinib (600 mg or 800 mg) delayed only thrombocyte recovery. Non-hematologic and non-infectious toxicity did not differ between the different combinations of imatinib and cytarabine.

The increased frequency of infectious complications that was noted in this series of patients treated with intermediate-dose cytarabine was most likely due to the prolonged period of neutropenia. Prolonged neutropenia is clearly associated with an increased risk of infectious complications.[16,17] Some additional mucosal toxicity and/or the placement of a central venous catheter may have contributed to the high number of patients with infectious complications after intermediate-dose cytarabine.[18,19] The frequency of fever of unknown origin was also increased, which is often observed during prolonged neutropenia and may also be related to the dose of cytarabine.[10,20]

Five of the 7 DLTs were accompanied by a streptococcal bacteremia. All occurred after the first cycle and especially after intermediate-dose cytarabine. Four patients with a streptococcal bacteremia had discontinued penicillin prophylaxis, which was according to protocol, and another patient with a streptococcal bacteremia did not receive penicillin but levofloxacine. The two toxic deaths, both with cerebral abscesses, were considered to be related to viridans streptococci. Serious complications associated with viridans streptococcal bacteremia are well known to occur in neutropenic patients with cancer receiving high-dose chemotherapy and are associated with a high mortality rate.[21] Severe oral mucositis after high dose chemotherapy is a major risk factor for these complications. Complications including acute respiratory distress syndrome, septic shock and renal failure are often described in these patients. Viridans streptococci are a common cause of brain abscesses in the literature, mostly occurring after otopharyngeal infections, endocarditis, or neurosurgical or dental procedures with secondary hematogenous spread.[22,23] No cases of cerebral abscesses have

been described in the literature after imatinib monotherapy. Cerebral edema has been reported as a rare complication of imatinib treatment.[24] However, no neurological symptoms indicating cerebral edema were present in these two patients prior to streptococcal septicemia, suggesting that the abscesses mainly resulted from streptococcal bacteremia.

A major molecular response was obtained in 30% of the patients shortly following combination therapy, which increased to 46% at 1 year. The initial molecular response rate obtained after combination therapy in our study seems promising. Longer follow-up is, however, needed to determine whether combination therapy increases the molecular response rate, prevents resistance, and to determine which patients benefit most. Preliminary results with combination therapy of imatinib and low-dose cytarabine in 30 patients with newly diagnosed CML in first chronic phase were reported by Gardembas et al.[25] At 1 year a complete hematologic response was observed in 97% of the patients and a complete cytogenetic response in 70% of the patients.[24] These results were comparable to our study and to those obtained with imatinib alone, but interestingly they also observed some early molecular responses.[24] Another important observation in the study of Gardembas was an increased hematologic CTC grade 3-4 toxicity of 53% and non-hematologic CTC grade 3-4 toxicity of 23% as compared to about 15% hematologic and 15-20% non-hematologic CTC grade 3-4 toxicity with imatinib alone (400 mg) .[4,5] In the present study hematologic toxicity CTC grade 3-4 was observed in nearly all patients and non-hematologic toxicity CTC grade 3-4 was observed in 36% of patients, which seems slightly more than in the French study. Collectively, both combination studies have demonstrated the feasibility of combining imatinib and cytarabine, but at the expense of enhanced toxicity as compared to imatinib alone. Toxicities with respect to infectious complications, hematologic and other toxicities are only acceptable if combination therapy would be associated with enhanced efficacy. However, mature follow-up of the combination of imatinib and cytarabine (either low- or intermediate-dose) is currently lacking and, therefore, it is not known whether combination therapy would prevent resistance and disease progression.

The present study was designed to explore the feasibility of imatinib and intravenous cytarabine and to obtain long-term efficacy of the different dose levels. Furthermore, our aim was to select a feasible, efficacious dose level associated with a high rate of molecular response, that could be explored further in a randomized study. While both dose levels of cytarabine met predefined feasibility criteria, the standard-dose of cytarabine (200 mg/m^2) seems preferable, because the higher dose of cytarabine (1000 mg/m^2) was associated with significantly more infectious complications. Increasing the dose of imatinib did not affect the feasibility of that combination. In addition, the combination of standard-dose cytarabine and imatinib may be given on an outpatient basis. Therefore, we selected a standard-dose of cytarabine (200 mg/m^2) together with high-dose imatinib (800 mg) to be compared with high-dose imatinib (800 mg) monotherapy for a subsequent randomized clinical trial, which was recently started.

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4

EFFICACY OF ESCALATED IMATINIB AND CYTARABINE IN NEWLY DIAGNOSED PATIENTS WITH CHRONIC MYELOID LEUKEMIA. RESULT OF THE DUTCH-BELGIAN HOVON-51 STUDY.

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Abstract

While most patients with first chronic phase CML obtain a complete cytogenetic response (CCR) upon treatment with a standard-dose of imatinib, complete molecular responses (CMR), however, are rare. Having reported feasibility previously, we hereby report efficacy of escalated imatinib in combination with 2 cycles of intravenous cytarabine as received by 162 CML patients included in the HOVON-51 study. With a median follow-up of 55 months, the 5-year cumulative incidences of a CCR, major molecular response (MMR), and CMR were 89%, 71%, and 53%, respectively. A higher Sokal risk score was inversely associated with CCR (HR = 0.63; 95% CI, 0.50-0.79, $P<.001$). A higher dose of imatinib and a higher dose of cytarabine were associated with increased CMR with HRs of 1.60 (95% CI, 0.96-2.68, $P=.07$) and 1.66 (95% CI, 1.02-2.72, $P=.04$), respectively. Progression-free survival and overall survival at 5 years were 92% and 96%, respectively. Achieving a MMR at 1 year was associated with complete absence of progression and a probability of achieving a CMR of 89%. It is concluded that the addition of intravenous cytarabine to imatinib as upfront therapy for CML patients is associated with a high rate of CMR. The study was registered at www.kankerbestrijding.nl as CKTO-2001-03.

Introduction

The introduction of imatinib, a specific kinase inhibitor of the BCR-ABL protein, in 1998 and its approval by the Food and Drug Administration in 2001, has dramatically changed prospects for patients with chronic myeloid leukemia (CML).[1,2] Most patients with newly diagnosed chronic phase CML nowadays achieve a complete cytogenetic response (CCR), which subsequently predicts for relatively long survival.[3] Moreover, patients achieving a major molecular response (MMR) do even better, as not a single patient that attained a MMR at 18 months had progressed at 5 years.[3,4] The recently presented 7-year follow-up data of the International Randomized Study of Interferon and STI571 (IRIS) study confirmed durability of cytogenetic responses and a low rate of progression.[5] However, the estimated 5-year event-free survival (EFS) was 83%, and an estimated another 16% of patients discontinued imatinib for various reasons within the first five years.[3] Comparable results were observed in a recent large single center study, with a 5-year probability of remaining in stable cytogenetic remission while still receiving first-line imatinib of 63%.[6] Collectively, these results indicate that although the majority of patients enter a stable cytogenetic remission, still more than one third of patients may be in need of an alternative therapy.

Patients needing second-line therapy include patients, who do not tolerate imatinib and patients acquiring resistance. Primary or acquired resistance against imatinib is currently defined at the hematologic, cytogenetic, and also molecular level.[7] It may be caused by different mechanisms, including point mutations in the *BCR-ABL* kinase domain, overexpression of *BCR-ABL*, additional chromosomal abnormalities in the Philadelphia (Ph) positive clone, and a relative insensitivity of quiescent leukemic stem cells to imatinib.[8-12] Prevention of resistance and improving the cytogenetic and molecular response rates may be achieved by different approaches, including dose escalation of imatinib, second-generation tyrosine kinase inhibitors, or combination therapy.[13-17] Several combinations have been explored in vitro and also in early clinical studies.[14,18-22] Among the combinations of imatinib and cytostatic drugs, the combination of cytarabine and imatinib was found to result in a synergistic effect, especially at higher concentrations of either drug.[20-22] Based on these findings, the HOVON cooperative study group set out to clinically explore the feasibility and efficacy of the combination imatinib and cytarabine applying a step-wise dose-increase of either drug. Recently, feasibility results of that combination were reported.[14] While a higher dose of cytarabine was associated with more infectious complications; a standard-dose of cytarabine appeared well tolerated. Here, efficacy of the combination of imatinib and intravenous cytarabine is reported with emphasis on the rate and duration of molecular responses as well as its major determinants.

Methods

The HOVON-51 was a multi-center study designed to investigate the feasibility and efficacy of escalated imatinib in combination with intravenous cytarabine in patients with early chronic phase CML. Inclusion criteria included: age between 18 and 65 years, presence of the Ph chromosome or *BCR-ABL* rearrangement, adequate organ function, registration within 6 months of diagnosis, and no previous treatment except for hydroxyurea. The ethics committees of all participating centers approved the study and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Patients were recruited as from August 2001 to November 2005.

Study design and treatment

The design of the study has been described in detail recently.[14] In short, patients were assigned to one of seven predefined, successive dose levels. Dose levels were open for inclusion, only when the preceding dose level had met the criteria of acceptable toxicity and safety, until finally the highest dose level V was evaluated. First, a pre-phase of imatinib (400 mg) monotherapy was given to all patients, followed by combination therapy of 2 cycles of intravenous cytarabine (200 mg/m^2 or 1000 mg/m^2 days 1 to 7) with imatinib (200 mg, 400 mg, 600 mg or 800 mg qd). Imatinib (400 mg, 600 mg or 800 mg) maintenance therapy was continued after the second cycle until disease progression, intolerance of treatment, or eligibility for allogeneic stem cell transplantation (allo-SCT), whichever occurred first.

Dose adjustments during imatinib maintenance therapy were made in case of non-hematologic toxicity of common toxicity criteria (CTC) grade 2 or higher. If a patient experienced a CTC grade 2 non-hematologic toxicity that did not resolve despite therapeutic intervention, imatinib was withheld until the toxicity had resolved to ≤ grade 1. If the toxicity CTC grade 2 recurred, imatinib was withheld and after resolution of the toxicity resumed at a reduced dose that was 100-200 mg qd lower than the dose used before the toxicity occurred. If ≥ CTC grade 3 recurred or if toxicity had been life threatening, treatment with imatinib was discontinued, and patients were taken off protocol. In case of hematologic toxicity ≥ CTC grade 4 during imatinib maintenance therapy, imatinib was withheld and resumed at the same or a reduced dose after resolution of the toxicity to ≤ grade 2. No dose adjustments were made for hematologic toxicity ≤ CTC grade 3.

Definition of end points

Hematologic and cytogenetic responses were as detailed before.[14] The definition of molecular response was adapted in order to be compatible with the international scale,[23,24] and defined as complete molecular response (CMR; no residual *BCR-ABL* transcripts by real-time quantitative polymerase chain reaction) or MMR (value of ≤ 0.1% on the international scale). Only *BCR-ABL* values resulting from assaying with a level of sensitivity of at least 0.01% were considered appropriate. If during follow-up cytogenetic results were not available, real-time quantitative polymerase chain reaction (RQ-PCR) measurement of *BCR-ABL* was used as a surrogate for CCR where *BCR-ABL* values below 1% were considered as achieving CCR.[15,25] Molecular response was centrally assessed at the Erasmus University Medical Center in Rotterdam using RQ-PCR on peripheral blood and/or bone marrow. Molecular analysis was done at baseline, after cycles 1 and 2, at 6 months, and at least every 3 to 6 months thereafter. All patients who failed to achieve a MMR at one year were evaluated for point mutations in the *ABL* kinase domain, which investigation was repeated during follow-up as long as patients failed to achieve a MMR. Patients, who lost their initial response or progressed during follow-up, were also evaluated for mutations. *BCR-ABL* mutation analyses was performed as previously described.[26]

Cumulative incidences of response are expressed as the time from registration to complete hematologic response (CHR), major cytogenetic response (MCR), CCR, MMR, and CMR. Loss of CHR was defined as a white blood cell count (WBC) > 20 x 10^9/l or progression to advanced phase CML; loss of MCR as an increase of Ph-positive metaphases by at least 30 percentage points to ≥ 35% Ph-positive metaphases; loss of CCR by the detection of one or more Ph-positive metaphases, loss of MMR as a 0.5-log increase of *BCR-ABL* to a *BCR-ABL* level > 0.1%; and loss of CMR as renewed detection of *BCR-ABL* transcript levels. In case of loss of hematologic, cytogenetic or molecular responses, confirmation by a subsequent evaluation at least 1 month apart was required. Progression was defined as development of accelerated phase or blast crisis CML, whichever came first. Failure on imatinib treatment was defined as progression (to advanced phase CML), loss of CHR, loss of MCR, or an increasing WBC (defined as doubling of the WBC to > 20 x 10^9/l on two occasions at least one month apart in a patient who had never attained a CHR despite receiving maximally tolerated doses of therapy).

Progression-free survival (PFS) was defined as the time from registration until progression or death, whichever came first. Failure-free survival (FFS) was defined as the time from registration until failure on imatinib treatment or death, whichever came first. Event-free survival was defined as the time from registration until failure on imatinib treatment, discontinuation of imatinib treatment, going off protocol treatment for any reason, or death, whichever occurred first. Overall survival (OS) was calculated as the time from registration until death whatever the cause. Patients still alive at the date of last contact were then censored.

Statistical methods

The cumulative incidences of CHR, MCR, CCR, MMR and CMR were calculated using competing risk analysis. Competing risks were disease progression, discontinuation of treatment before achieving response, or death without previous response. As an allo-SCT was allowed as off protocol treatment if no cytogenetic response was acquired within 12 months or according to the physician's preference, those patients were censored at the date of allo-SCT. Progression-free survival, EFS, FFS, and OS were estimated by the Kaplan-Meier method, and 95% confidence intervals (CIs) were determined. Patients who received an allo-SCT were censored at the date of transplantation. Time to response and survival end points were illustrated by Kaplan-Meier curves until 5 years.[27] In our trial, patients had been assigned to receive standard- or intermediate-dose cytarabine (200 mg/m^2 and 1000 mg/m^2), as well as low/standard-dose (200 and 400 mg) or high-dose (600 mg and 800 mg) imatinib. Univariate and multivariate Cox regression analyses,[28] without and with interaction terms, were performed to evaluate the effect of higher dose levels and the impact of the Sokal risk score and Euro score on clinical outcome. Hazard ratios (HRs) with 95% CIs were determined. All reported *P* values are 2 sided, and a significance level $\alpha = .05$ was used.

Results

Patient characteristics are presented in Table 1. Median age at diagnosis was 47 years (range: 19-65 years); patients were fairly evenly distributed among the 3 Sokal risk categories. A flow diagram of treatment actually received is depicted in Figure 1. One hundred and sixty-two patients received a first cycle of combination therapy and 140 patients (86%) also received a second cycle of combination therapy. One hundred fifty-seven patients (97%) started with imatinib maintenance therapy. The current analysis is based on data collected up to December 18, 2008, resulting in a median follow-up of 55 months (range: 10-84 months). Currently, 112 patients (69%) are still on protocol treatment, and 50 patients went off protocol treatment for various reasons (Figure 1). Second-line therapy included allo-SCT from either a related or a matched unrelated donor in 18 patients, nilotinib or dasatinib in 14 patients, chemotherapy in 7 patients, and other treatment modalities in 7 patients.

Table 1. *Baseline characteristics of all patients*

Characteristic	All patients
	(N=162)
Age at diagnosis, y	
median	47
range	19-65
Sex, no. (%)	
male	95 (59%)
female	67 (41%)
Sokal risk group, no. (%)	
low (< 0.8)	59 (36%)
intermediate (0.8-1.2)	50 (31%)
high (> 1.20	43 (27%)
unknown	10 (6%)
Euro score, no. (%)	
low (≤ 780)	70 (43%)
intermediate (> 780-1480)	57 (35%)
high (> 1480)	23 (14%)
unknown	12 (7%)
Dose of imatinib, no. (%)	
low/standard-dose (200 mg and 400 mg)	49 (30%)
high-dose (600 mg and 800 mg)	113 (70%)
Dose of cytarabine, no. (%)	
standard-dose (200 mg/m^2)	107 (66%)
intermediate-dose (1000 mg/m^2)	55 (34%)

Percentages may not sum up to 100% due to rounding

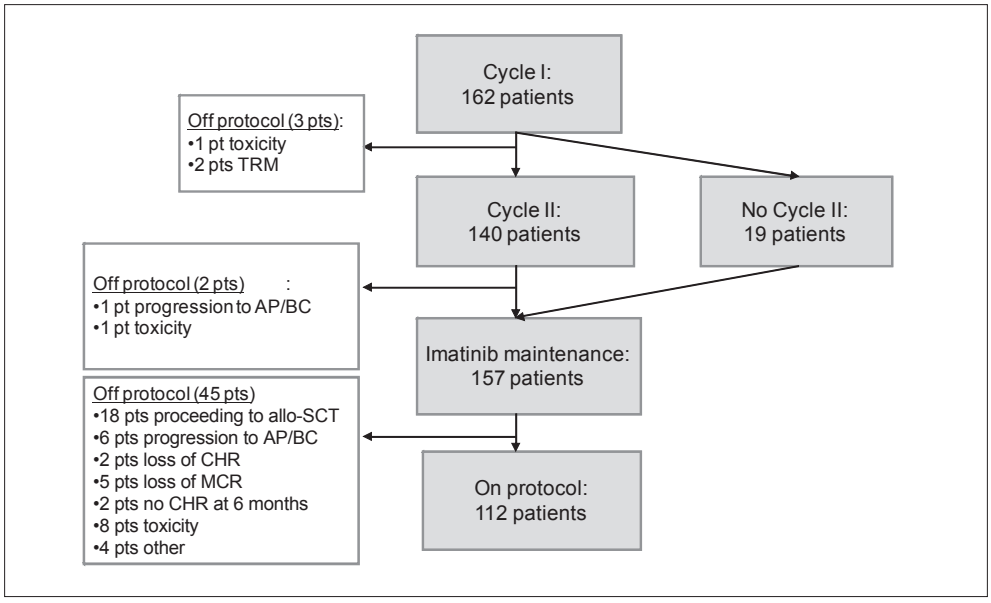

Figure 1. *Flow diagram of 162 patients entered in the Dutch-Belgian HOVON-51 study. Reasons for going off protocol treatment are indicated*

Hematologic, cytogenetic, and molecular response

Patient responses are presented in Table 2, and Figures 2, 3, and 4. One hundred fifty-four patients developed a CHR, 146 patients a MCR, and 135 patients a CCR. The median time to a CCR was approximately 4.5 months. In total, 107 patients achieved a MMR, and 78 patients developed a CMR on protocol treatment. The median time to MMR was 11 months and the median time to CMR was approximately 22 months. With a median follow-up of 55 months, 9 patients lost their CHR, 16 patients lost their previously established MCR and 17 patients lost their CCR. Of all 107 patients with a MMR, 6 patients lost that response, and loss of CMR was observed in 10 patients (Table 2). At 5 years, cumulative incidences of a CCR, MMR, and CMR were, respectively, 89%, 71%, and 53% (Figure 2A,B,C). Of note, 89% of the patients who achieved a MMR at 1 year subsequently developed a CMR. Furthermore, none of the 107 patients with a MMR subsequently progressed to accelerated phase or blast crisis CML. Four out of 135 patients with a CCR progressed to accelerated phase or blast crisis, while 5 out of 27 patients who failed to achieve a CCR also progressed to advanced phase CML. Among the 103 patients with a CCR at one year, 91 (88%) subsequently obtained a MMR and 71 patients (69%) ultimately developed a CMR. In contrast, molecular responses were rare in patients failing to achieve a CCR at 1 year. Among 41 patients continuing protocol treatment, but who failed to achieve a CCR at one year, 27 patients (66%) subsequently developed a CCR at later time points (Figure 2A), 15 patients (37%) attained MMR, and only 6 patients (15%) ultimately developed a CMR.

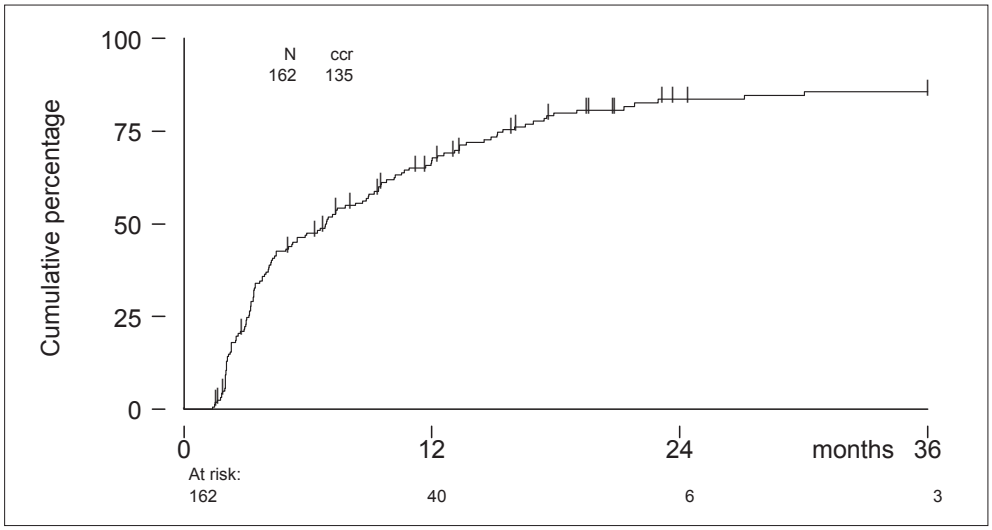

Figure 2A. *Cumulative incidence of complete cytogenetic response.*

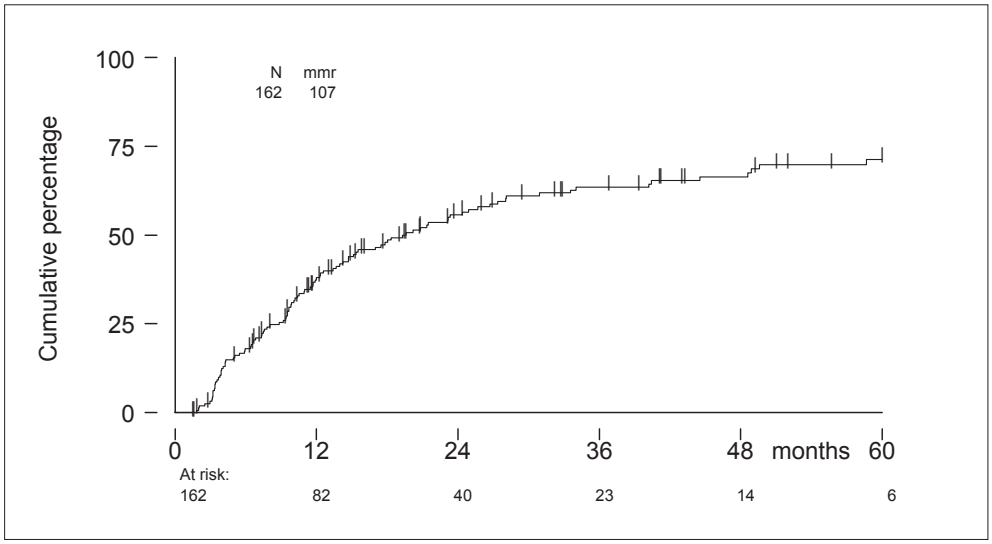

Figure 2B. *Cumulative incidence of major molecular response.*

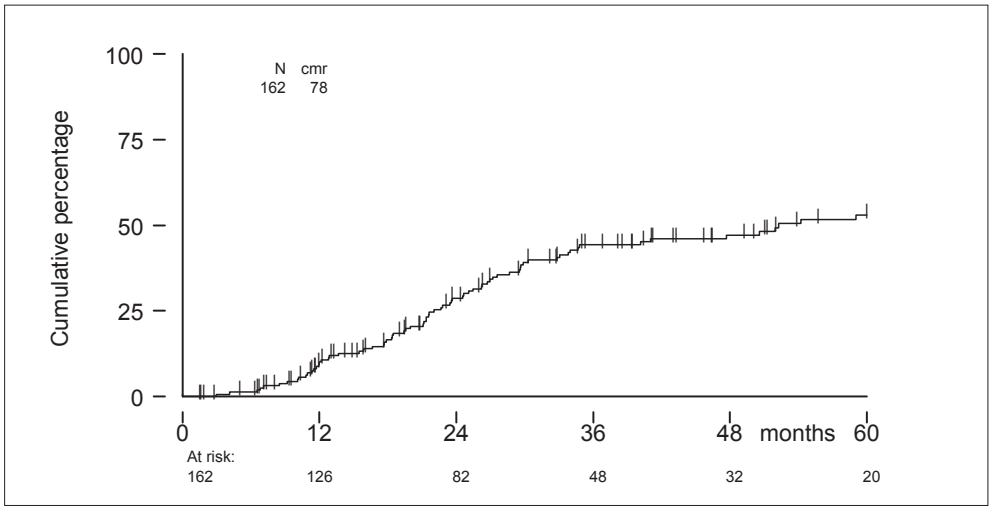

Figure 2C. *Cumulative incidence of complete molecular response.*

Table 2. *Patient responses (N=162)*

Type of Response	No.
CHR	
No	8
Progression to AP/BC	2
Yes	154
Loss of CHR	9
Progression to AP/BC	7
CCR	
No	27
Yes	135
Loss of CCR	17
Loss of CHR	5
Progression to AP/BC	4
MMR	
No	55
Yes	107
Loss of MMR	6
Loss of CCR	1
Loss of CHR	1
Progression to AP/BC	-
CMR	
No	84
Yes	78
Loss of CMR	10
Loss of MMR	2
Loss of CCR	-
Loss of CHR	-
Progression to AP/BC	-

Abbreviations: CHR, complete hematologic response; AP/BC, accelerated phase or blast crisis; CCR, complete cytogenetic response; MMR, major molecular response; CMR, complete molecular response

Table 3. *Results of the multivariate analysis*

Parameter	Major cytogenetic response		Complete cytogenetic response		Major molecular response		Complete molecular response	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Dose of cytarabine	1.08 (0.75-1.57)	.66	1.02 (0.69-1.51)	.91	1.16 (0.75-1.78)	.50	1.66 (1.02-2.72)	.04
Dose of imatinib	1.07 (0.74-1.55)	.73	1.38 (0.93-2.04)	.11	1.67 (1.06-2.61)	.03	1.60 (0.96-2.68)	.07
Sokal risk score	0.56 (0.45-0.70)	< .001	0.63 (0.50-0.79)	< .001	0.74 (0.58-0.96)	.02	0.90 (0.67-1.22)	.51

Different parameters were evaluated for prediction of response, including Sokal risk score, Euro score, dose of imatinib, and dose of cytarabine. There were significant differences in the rates of MCR and CCR among patients, according Sokal risk and Euro score in univariate analysis. A higher Sokal risk score remained adversely associated with MCR and CCR (HR = 0.63; 95% CI, 0.50-0.79, $P < .001$) (Table 3) in multivariate analysis. At 1 year the cumulative incidences of a CCR was 76% in patients with a low Sokal score, 74% in patients with an intermediate Sokal score, and 40% in patients with a high Sokal score. However, at 5 years these differences in response rates were less pronounced with 89%, 93%, and 81%, in low, intermediate, and high-risk patients, respectively. Furthermore, a higher Sokal score was also inversely associated with MMR (HR = 0.74; 95% CI, 0.58-0.96, $P = .02$) (Table 3), but not with CMR. In contrast, the dose of imatinib and the dose of cytarabine were not associated with cytogenetic response, but a higher dose of imatinib (600 or 800 mg) appeared to be associated with a better MMR and CMR (HR = 1.60; 95% CI, 0.96-2.68, $P = .07$) (Table 3, Figure 3). Independently, also the higher dose of cytarabine was associated with a better CMR. Sixty percent of patients, receiving the higher dose of cytarabine, developed a CMR at 5 years as compared to 50% for patients receiving a standard-dose of cytarabine (HR = 1.66; 95% CI, 1.02-2.72, $P = .04$) (Table 3, Figure 4).

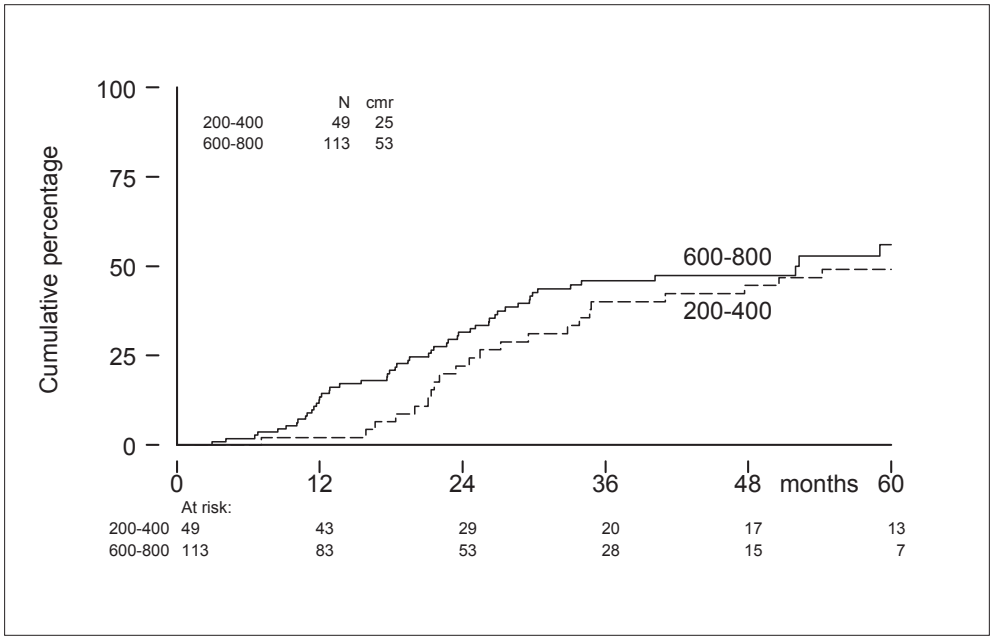

Figure 3. *Cumulative incidences of complete molecular response by dose of imatinib (HR = 1.60; 95% CI, 0.96-2.68, P = .07)*

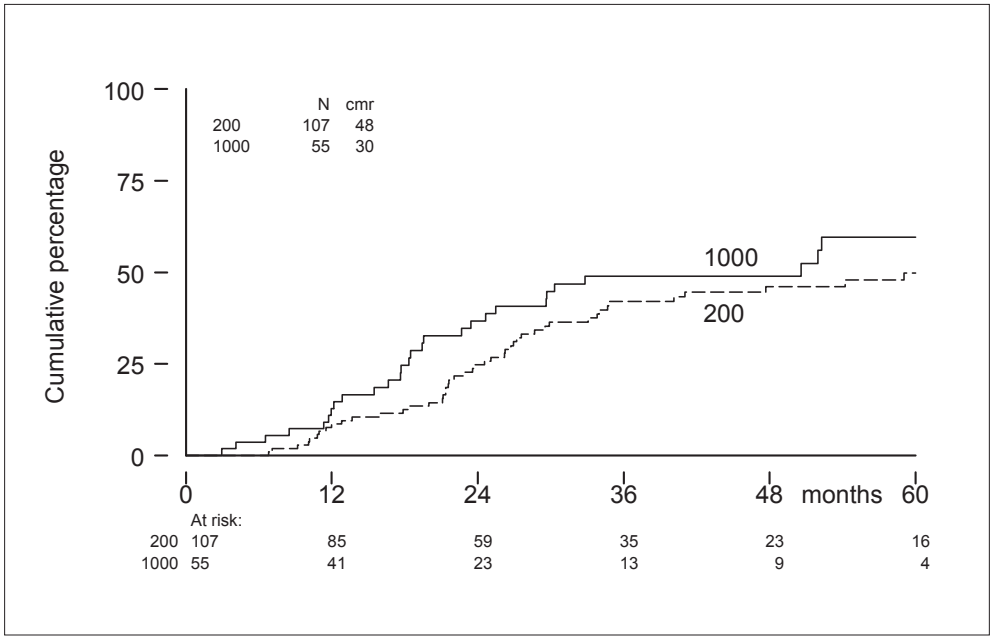

Figure 4. *Cumulative incidences of complete molecular response by dose of cytarabine (HR = 1.66; 95% CI, 1.02-2.72, P = .04)*

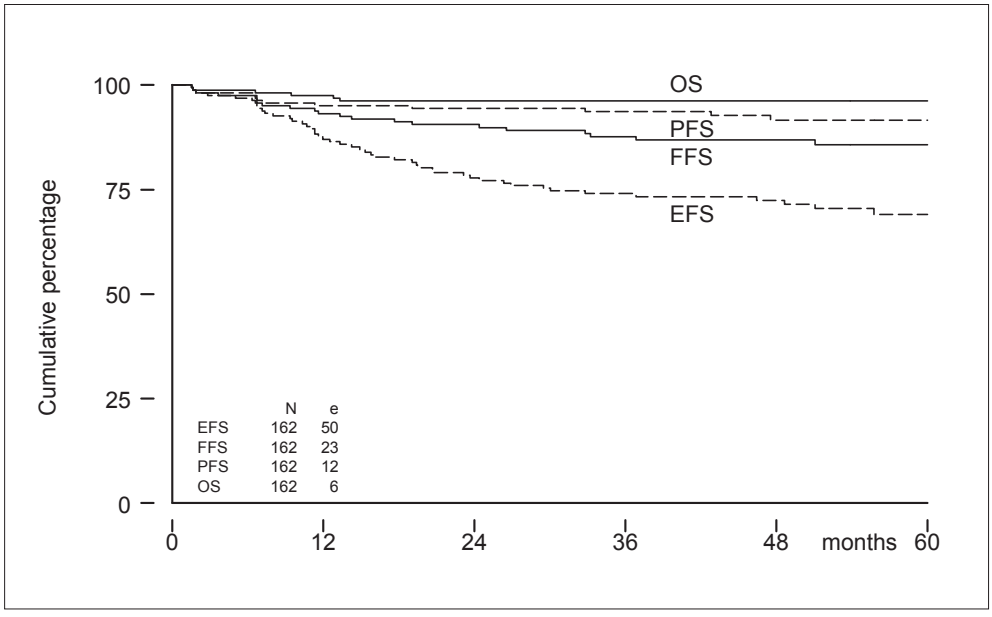

Figure 5. *Event-free survival (EFS), failure-free survival (FFS), progression-free survival (PFS), and overall survival (OS)*

Progression-free, overall, failure-free, and event-free survival

After a median follow-up of 55 months, 9 patients have developed accelerated phase or blast crisis CML and a total of 3 patients died resulting in a 5-year PFS of 92% (95% CI, 85%-95%) (Figure 5). The estimated annual rate of progression was 5.0% in the first year, 0.7% in the second year, 0.8% in the third year, 2.2% in the fourth year, and 0% in the fifth year. Due to the limited number of events, prognostic factors were not evaluated for possible association with PFS. In total, 6 patients died, resulting in an OS at 5 years of 96% (95% CI, 92%-98%). Causes of death of these 6 patients included blast crisis CML in 3 patients, excessive toxicity in 2 patients and an unrelated cause in 1 patient. Recipients of allogeneic stem cell graft were censored at the time of transplant for the latter analysis in concordance with earlier reports and to facilitate comparison.[3,9] Twenty-seven patients ultimately received an allo-SCT in second or third line, predominantly because of primary or secondary resistance (Table 4). Twelve out of these 27 patients died due to either non-relapse mortality (n=11) or progressive disease (n=1). Survival without censoring of allogeneic SCT recipients at the time of transplant estimated 88% at 5 years.

Table 4. *Indications and outcome of patients after allogeneic hematopoietic stem cell transplantation*

Variable	Allo-SCT patients
	(N=27)
Indication allo-SCT	
Primary resistance, failure to achieve:	
CHR at 6 months	2
MCR at 12 months	6
Secondary resistance:	
progression to AP/BC	4
increasing WBC	1
loss of MCR	4
Intolerance of imatinib	3
Physicians preference	7
Outcome	
Alive	15
Death	
CML (BC)	1
Non-relapse mortality	
graft versus host disease	4
infections	5
other	2

Abbreviations: CHR, complete hematologic response; MCR, major cytogenetic response; AP, accelerated phase; BC, blast crisis; WBC, white blood cell count; allo-SCT, allogeneic stem cell transplantation; CML, chronic myelogenous leukemia

Failure-free survival was defined as failure on imatinib treatment including progression to advanced phase CML, loss of CHR, loss of MCR, or an increasing WBC for patients without CHR, or death. Twenty patients failed imatinib treatment, of which 7 patients progressed to the accelerated phase or blast crisis as first event of treatment failure, 2 patients had an increasing WBC, 2 patients had a loss of CHR, and 9 patients had a loss of MCR. Another 3 patients died without prior failure on imatinib treatment. The estimated 5-year FFS was 86% (95% CI, 79%-91%) (Figure 5). Neither the dose of cytarabine or imatinib, nor Sokal risk and Euro score were associated with FFS. However, time dependent analysis showed that early cytogenetic and molecular responses had a favorable impact on FFS. A landmark analysis of the 103 patients who had achieved a CCR at 1 year revealed a superior estimated 5-year FFS of 97% as compared to 77% in 41 patients without a CCR at 1 year ($P < .001$) (Figure 6A). Moreover, the 5-year FFS for 61 patients who rapidly achieved a MMR at 12 months was superior as compared to the 83 patients who had failed to attain a MMR at 1 year (100% versus 86%; $P = .002$) (Figure 6B).

Furthermore, also EFS was assessed, with EFS defined as all failures and including patients going off protocol for other reasons than progression, including intolerance of treatment and allo-SCT. A total number of 50 events were noted, resulting in 5-year EFS of 69% (95% CI, 61%-76%) (Figure 5). A higher Sokal risk and Euro score were associated with worse EFS in univariate analysis. A higher Sokal risk score remained adversely associated with EFS (HR = 1.55; 95% CI, 1.08-2.22, $P = .02$), when adjusted for dose of cytarabine and imatinib.

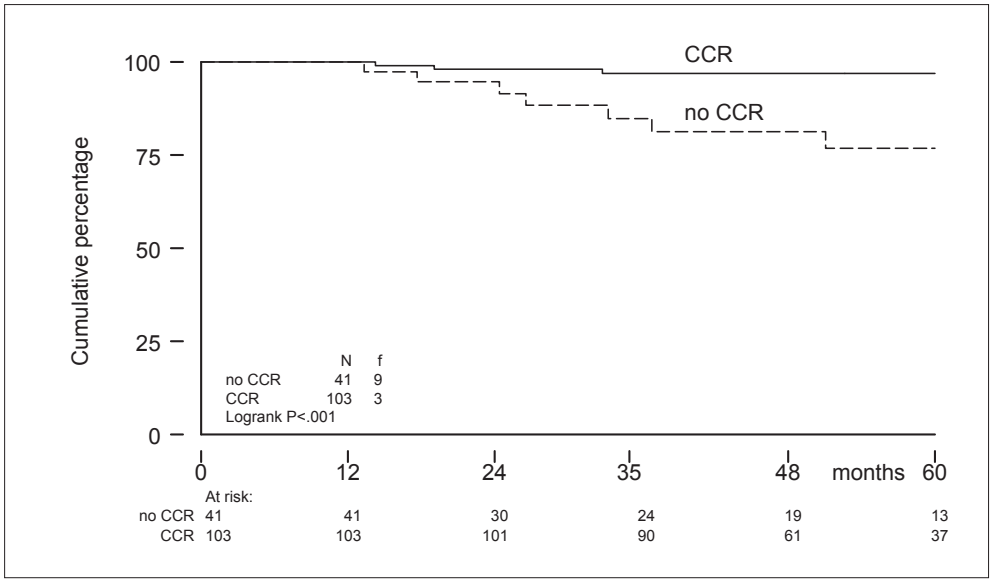

Figure 6A. *Landmark analysis of failure-free survival (FFS), by complete cytogenetic response at 1 year*

Point mutations in the *BCR-ABL* kinase domain

Patients failing to achieve a MMR at one year, and at subsequent evaluation time points thereafter were evaluated for point mutations within the *BCR-ABL* kinase domain. In addition, patients with primary or secondary hematologic or cytogenetic resistance and all patients who, at any time, progressed to accelerated phase or blast crisis were evaluated for mutations. In total, 153 samples were evaluated for point mutations in the kinase domain. It resulted in a cumulative incidence of mutations of 10% at 5 years. In total, 14 different mutations were detected in 15 patients (Table 5), including 2 patients with a T315I mutation. Nine out of these 15 patients with a mutation subsequently lost their response, and 3 patients progressed to advanced phase CML.

Tolerance of protocol treatment

Adverse events and side effects during the phase of combination therapy were reported in detail before.[14] During maintenance, the most frequent adverse events CTC ≥ grade 2 included constitutional symptoms (34%) and gastrointestinal complaints (33%), however, toxicity CTC grade 3 or 4 occurred infrequently. Both the incidence and severity of these side effects were essentially similar to what can be observed in patients receiving monotherapy with imatinib as reported before.[3] Combination therapy and maintenance were well tolerated as illustrated by only 9% of patients discontinuing treatment because of side effects (n=15), which represent all discontinuations including the toxic deaths), an EFS of 69%, and a total number of 112 patients still continuing protocol treatment.

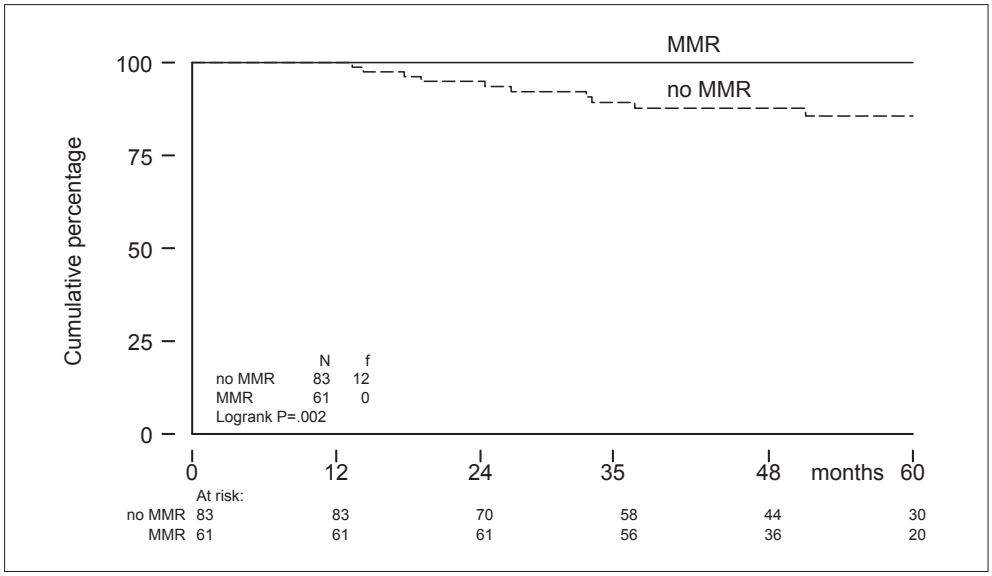

Figure 6B. *Landmark analysis of failure-free survival (FFS), by major molecular response at 1 year*

Table 5. *BCR-ABL kinase domain mutations*

Type of mutation	Associated resistance	Cytogenetic abnormalities
K247R	primary resistance: no CHR at 6 months	46,XY,t(9;22)(q34;q11) [15] / 47, idem,+8 [7]
G250E	BC	50,XX,+3,inv(3)(q21q26)x2,+8, t(9;22)(q34;q11), +12,+der(22)t(9;22)(q34;q11) [14]
Y253H	BC	-
E279K	loss of CCR	-
T315I	primary resistance: no MCR at 12 months	-
T315I	primary resistance: no MCR at 12 months	-
F317L	loss of CCR	-
M351T	loss of CCR	-
F359V	increasing WBC	-
V371A	no resistance	-
G383G	loss of MMR	-
H396P	no resistance	-
E450K	BC	46, XY,-7,+8,t(9;22)(q34;q11) [26]
E459K	loss of MCR	-
P480L	no resistance	-

Abbreviations: CHR, complete hematologic response; MCR, major cytogenetic response; CCR, complete cytogenetic response; MMR, major molecular response; MR, molecular response; BC, blast crisis; WBC, white blood cell count

Discussion

Allogeneic hematopoietic SCT is still the only treatment modality that has been proven to cure patients with CML.[29] Although imatinib treatment is associated with high rates of cytogenetic responses and major molecular responses in patients with first chronic phase CML, complete molecular responses are rare and the majority of patients continuously harbour minimal residual disease, necessitating prolonged treatment with imatinib.[3,4,6] With the ultimate aim to improve the CMR rate, the HOVON study group set out to explore the feasibility and efficacy of the combination of cytarabine and imatinib. Recently, the feasibility of intravenous cytarabine and escalated imatinib was reported.[14] With a median follow-up of 55 months, long-term efficacy of combination therapy is presented here. The most important findings of our study include a high CMR rate, a relatively low incidence of primary cytogenetic and molecular resistance, resulting in a high PFS, and a relatively high number of patients still continuing protocol treatment, while maintaining their remission.

The cumulative incidence of a CMR was 53% at 5 years. To what extent can that percentage be explained by the addition of cytarabine? A higher dose of imatinib monotherapy may already be associated with a faster and better response, although different results were observed in distinct risk-categories of patients.[13,30-34] In addition, the association between plasma trough levels and outcome, as was observed by several investigators,[35,36] has suggested that a threshold of at least 1002 ng/ml should be aimed for, but it is unclear to what extent responses may be further improved when higher levels of imatinib are achieved. A modest dose dependent effect of imatinib was also apparent in our study (Table 3, Figure 3), which was of borderline significance. It seems therefore unlikely that the higher dose of imatinib per se has resulted in the observed CMR rate. Rather, the earlier observed in vitro synergistic or additive effect of cytarabine[20-22] seems to be mirrored here clinically. An additive effect of cytarabine is further supported by the significant dose dependent effect of cytarabine as observed in our study. Moreover, none of the patients receiving the higher dose of cytarabine has shown progressive disease until the latest follow-up. While our results do suggest at least an additive effect of cytarabine, however, only a direct comparison in a prospective randomized trial may exactly answer the question to what extent cytarabine improves the molecular response rate and prevents disease progression. To that end, the HOVON cooperative group recently started a prospective randomized study comparing 800 mg imatinib versus the combination of 800 mg combined with cytarabine at a dose of 200 mg/m^2 intravenously. Two other cooperative groups explored the combination of cytarabine and imatinib. The French cooperative groups demonstrated the feasibility of imatinib and low-dose cytarabine,[18] but long-term results are not available yet. Furthermore, the Australian cooperative group developed a protocol including addition of cytarabine for patients failing to obtain a sufficient response 3 months after dose escalation of imatinib. Recently, their results were reported and it appeared that only a minority of patients actually received the combination, which precludes any definite conclusion as regards the additive value of cytarabine in their study.[15]

By inducing a high CMR, combination therapy may prevent primary resistance at the various levels (hematologic, cytogenetic, molecular), and it may also prevent secondary resistance in patients relapsing from an earlier established response. To what extent was resistance prevented? Primary cytogenetic resistance defined as failure to achieve a CCR at 18 months, was observed in 36 patients (22%) in our study. Nineteen out of the 36 patients (53%), who failed to achieve a CCR by 18 months, were Sokal high-risk patients. While a percentage of 22% may be somewhat better as can be observed following imatinib only (approximately 30% in the Hammersmith study[6]), still primary cytogenetic resistance is of concern and combination therapy only partially prevented cytogenetic resistance. It indicates that a subset of high-risk patients is still in need of a more efficient approach. Furthermore, additional parameters apart from those incorporated in the Sokal and Euro scores may be needed to more accurately identify the patients at highest risk of primary cytogenetic resistance. New diagnostic techniques such as gene expression profiling and single nucleotide polymorphisms may possibly add to the well established risk scores.[37,38] Secondary resistance

percentages were rather low and PFS estimated 92% at 5 years, as compared to an estimated 5-year PFS of 83% in the Hammersmith study.[6] As outlined by de Lavallade et al, another important outcome estimate is the 5-year probability of achieving and maintaining a MCR, while continuing imatinib. It was 63% for patients with early chronic phase CML receiving a standard-dose of imatinib in the Hammersmith series of patients.[6] For comparison, 69% of the patients in the present study maintained at least an earlier established MCR and were still on imatinib according to protocol. Apart from an encouraging efficacy of combination therapy, that high percentage of patients continuing protocol treatment also illustrates that combination therapy was rather well tolerated.

Our intention was to improve the molecular response rate. What is the advantage of achieving a major and/or complete molecular response? Our results, as well as those by several others, clearly suggest that patients with a more pronounced response, such as a MMR, benefit in terms of a lower risk of disease progression and prolonged PFS.[3,4,39-41] Therefore, aiming for a MMR has been advocated as an important treatment goal by several investigators.[4,39] Is a further improvement up to the level of a CMR of additional benefit? Recent preliminary reports have suggested that a subset of patients in continued CMR may potentially be cured, as was suggested by absence of molecular relapse following cessation of imatinib maintenance. Rousselot et al. initially described 12 patients who discontinued imatinib with undetectable residual disease for more than 2 years.[42] Six patients were able to discontinue imatinib without disease recurrence. A more recent follow-up and inclusion of a total of 50 patients essentially showed the same picture with approximately 50% of patients maintaining PCR-negativity after cessation of imatinib.[43] A similar observation was done in Australia, with a relatively high failure free survival, but longer follow-up may be needed to definitely assess to what extent patients may be cured.[44] Collectively, these studies for the first time suggest that apart from allo-SCT, also "conventional" treatment based on tyrosine kinase inhibition may possibly be associated with cure. It is therefore, that the HOVON cooperative group has amended the present HOVON-51 study, by allowing all patients in continued CMR of at least 2 years to take part in a prospective study evaluating stopping imatinib maintenance. Thereby, we may be able to more definitely assess the contributing value of cytarabine in terms of possible cure. It should, however, be emphasized, that interruption and stopping of tyrosine kinase inhibitor therapy is experimental and should only be explored within the context of well-monitored prospective studies.

In conclusion, following earlier in vitro findings,[20-22] our clinical results may mirror the contributing effect of cytarabine to that of imatinib in patients with first chronic phase CML. The additional value of cytarabine in first chronic phase CML seems a better eradication of residual disease as reflected by a relatively high rate of CMR. While cytogenetic resistance may partially be prevented, a subset of high-risk patients still represents a category of patients for which better approaches are needed. The ultimate advantage of an increased CMR rate should be assessed in future studies, including well-monitored studies evaluating cessation of imatinib in patients with a long-lasting CMR.

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POLYMORPHISMS IN THE MULTIDRUG RESISTANCE GENE MDR1 (ABCB1) PREDICT FOR MOLECULAR RESISTANCE IN PATIENTS WITH NEWLY DIAGNOSED CHRONIC MYELOID LEUKEMIA (CML) RECEIVING HIGH-DOSE IMATINIB.

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Abstract

Hematologic and cytogenetic responses to first-line imatinib are high in patients with chronic myeloid leukemia (CML), but only a minority of patients proceeds to a complete molecular response (CMR). Persistent residual disease at the molecular level may be designated as molecular resistance and has been suggested to be accounted for by quiescent malignant stem cells. P-glycoprotein (P-gp), which is encoded by the ABCB1 multi drug resistance (MDR1) gene, has been demonstrated to mediate efflux of imatinib. As hematopoietic progenitor cells efficiently express P-gp, it was hypothesized that single nucleotide polymorphisms (SNPs) of the ABCB1 gene that account for differences in functional activity may possibly be involved in the probability of developing a molecular response to imatinib and molecular resistance. Therefore, we set out to evaluate whether the 3 most prevalent MDR1 SNPs would be associated with molecular response in a cohort of 46 CML patients receiving 800 mg imatinib. The 3 MDR1 SNPs (C1236T; G2677T/A; C3435T) proved to be in strong linkage disequilibrium ($P<.001$). Patient characteristics were distributed evenly among groups of patients, classified according to SNP-genotype. The probability of developing either a major molecular response (MMR) or CMR proved to depend strongly on SNP-genotype, which remained significant following multivariate analysis. Patients homozygous for allele 3435T showed a CMR rate of 10% versus a CMR of 50% at 1 year in patients homozygous for allele 3435C (HR: 0.24 (0.07-0.83, $P=.04$). Hazard ratio's associated with C1236T and G2677T were 0.27 (0.08-0.97, $P=.01$) and 0.23 (0.06-0.88, $P=.05$), for the 1236TT genotype versus the 1236CC genotype and 2677TT genotype versus the 2677GG genotype, respectively. Given the linkage disequilibrium, it remained unclear which individual SNP accounted for that difference. It is concluded that molecular resistance in CML patients receiving high-dose imatinib is strongly associated with ABCB1 genes polymorphisms, suggesting a role for P-gp mediated drug efflux in malignant hematopoietic stem cells, that account for persistent residual disease.

Introduction

Imatinib mesylate has been a major breakthrough in the treatment of chronic myeloid leukemia (CML). Most patients currently develop a complete hematologic response (CHR) and complete cytogenetic response (CCR) to imatinib.[1] Primary hematologic and cytogenetic resistance occurs in 5% and 15-20% of patients, respectively, receiving upfront imatinib therapy in first chronic phase.[2,3] Underlying mechanisms of hematologic and cytogenetic resistance include (among many mechanisms) pharmacokinetics, point mutations of the *ABL* kinase domain, and additional cytogenetic aberrations.[4,5] Pharmacokinetic resistance resulting in insufficient plasma levels of imatinib plays an important role, as it was reported that the probability of developing a CCR depends on the steady state plasma level achieved.[6,7] Plasma levels following a standard-dose of imatinib may be influenced by a variety of mechanisms including drug uptake, distribution, metabolization and excretion. While resistance at the hematologic and cytogenetic level has been studied more elaborately, resistance at the molecular level is still poorly understood. Persistent residual disease, which can be monitored by sensitive quantitative polymerase chain reaction (Q-PCR)-techniques, has been suggested to be accounted for by residual malignant hematopoietic stem or progenitor cells, that are relatively insensitive to imatinib.[8,9] Point mutations and/or cytogenetic abnormalities have, however, only rarely been observed in hematopoietic progenitor cells from patients with persistent disease at the molecular level. Recently, it was shown that OCT-1 activity, a mechanism involved in imatinib uptake, was an important determinant of molecular response.[10] Among possible other mechanisms involved, a role for P-glycoprotein (P-gp) mediated efflux of imatinib has been suggested before, but results from different clinical studies showed conflicting results.[11,12]

P-glycoprotein, encoded for by the ABCB1 multiple drug resistance gene (MDR1), belonging to the ATP-binding cassette (ABC) membrane transporter gene subfamily, is a multispecific efflux transporter of many drugs and xenobiotics.[13,14] Experimentally, overexpression of P-gp and subsequent inhibition, has unequivocally been demonstrated to strongly influence intracellular imatinib concentrations.[15-19] In addition, in vitro studies also demonstrated that cell kill of BCR-ABL positive cell lines may depend on intracellular imatinib concentrations, as can be influenced by P-gp and by inhibitors of P-gp .[16,17] Numerous single nucleotide polymorphisms (SNPs) have been identified in the ABCB1 gene and predominating haplotypes were subsequently demonstrated in different ethnic groups.[20,21] The SNPs 1236C>T, 2677G>T/A, and 3435CT are the most common variants in the coding region of ABCB1[21] and have been associated with altered drug levels and difference of response for numerous diseases[22] Only few study groups have evaluated the role of these SNPs in CML so far. Results by Duluq et al.[12] recently suggested higher molecular response rates in patients with the 1236TT genotype, but findings by Gurney et al.[11] rather suggested that the TT genotype is associated with enhanced clearance of imatinib and reduced response rates.

We recently evaluated results from a phase II study in first chronic phase CML patients, exploring escalating dosages of imatinib given in conjunction with iv cytarabine.[23,24] An encouraging rate of molecular response was observed, especially in patients receiving a higher dose of imatinib, although molecular resistance in patients receiving 800 mg imatinib was still approximately 40%.[24] The ABCB1 gene and its SNPs may theoretically affect the molecular response either by overall pharmacokinetic resistance by influencing plasma levels of imatinib, or alternatively, by promotion of drug-efflux from malignant hematopietic stem or progenitor cells. In order to preferentially study the latter mechanism, it was our intention to solely focus on patients receiving the higher 800 mg dose of imatinib to avoid variance of plasma levels due to different drug dosing and because the majority of 800 mg patients may be expected to achieve a sufficient trough level of imatinib. In addition, patients receiving 800 mg of imatinib constituted the majority of patients in the HOVON study.[23] Thus, given the current inconsistent findings in literature and the incompletely understood nature of molecular resistance in CML, we set out to evaluate whether the 3 most common SNPs of the ABCB1 gene would predict for molecular response or primary molecular resistance in a cohort of 46 first chronic phase CML patients receiving 800 mg imatinib.

Methods

Study design

Forty-six patients qualified for the present study, as these patients received 800 mg imatinib continuously, gave their informed consent, and had DNA-samples available. These patients had received 800 mg imatinib for a median time period of 45 months (range, 7-60 months) and they all participated in the HOVON-51 study.[23,24] As part of that protocol, patients also received 2 courses of iv cytarabine (200 mg/m^2 or 1000 mg/m^2, day 1-7), which were given at the start of treatment. Pharmacokinetics of cytarabine are not affected by ABCB1 gene SNPs, as cytarabine is not a substrate for the P-gp efflux pump.[25]

DNA and RNA preparation and molecular analysis

RNA was isolated from white blood cells that were purified from bone marrow or peripheral blood by centrifugation after red cell lysis. The white blood cells were lysed in RNA-Bee solution (Bio-Connect, Huissen, The Netherlands) and RNA was isolated according to the manufacturers protocol. The RNA purity and quality were verified by the 260/280 ratio and agarose gel electrophoresis. Genomic DNA was isolated either automatically using the MagNa Pure LC System (Roche, Woerden, The Netherlands) or manually by high salt isolation or columns with the ZR Genomic DNA II Kit (Zymo Research, Orange, CA, USA).

Genotype analyses

The MDR-1/ABCB1 genotyping was done using TaqMan allelic discrimination assays on an ABI Prism 7000 Sequence detection system. Each assay consisted of two allele-specific minor groove binding (MGB) probes, labeled with the fluorescent dyes VIC and FAM. The primer and probe sequences of the Assay-by-Design assays (Applied Biosystems) are listed in Table 1. For the tri-allelic MDR-1/ABCB1 variant G2677A/T, two separate assays were designed, one detecting the 2677G>A SNP and one detecting the 2677G>T polymorphism. Polymerase chain reactions were performed in a reaction volume of 12.5 µl, containing assay-specific primers, TaqMan Universal PCR Master Mix No AmpErase UNG (2X) and 12.5 ng genomic DNA. The thermal profile consisted of an initial incubation at 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds, and an annealing and extension step at 60°C for 1 minute. Genotypes were scored by measuring allelic-specific fluorescence using the SDS 1.2.3 software for allelic discrimination (Applied Biosystems).

Definition of end points

Molecular responses have been defined previously.[24] The definition of molecular response was adapted in order to be compatible with the international scale,[26] and defined as CMR (no residual *BCR-ABL* transcripts by RQ-PCR) or MMR (value of ≤ 0.1% on the international scale). Only *BCR-AB*L values resulting from assaying with a level of sensitivity of at least 0.01% were considered appropriate. Primary molecular resistance was defined by absence of a MMR at 24 months of therapy and by absence of a CMR at 36 months from the start of treatment.

Table 1. *Primer and probe sequence of the Assay-by-Design assays*

SNP	Primer /Probe	Sequence
MDR-1 1236C>T	Forward primer	TCTCACTCGTCCTGGTAGATCTTG
	Reverse primer	CACCGTCTGCCCACTCT
	VIC probe	TCAGGTTCAGGCCCTT
	FAM probe	TCAGGTTCAGACCCTT
MDR-1 2677G>A	Forward primer	AATACTTTACTCTACTTAATTAATCAATCATATTTAGTTTGACTCA
	Reverse primer	GTCTGGACAAGCACTGAAAGATAAGA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	CTTCCCAGTACCTTC
MDR-1 2677G>T	Forward primer	CTTAGAGCATAGTAAGCAGTAGGGAGT
	Reverse primer	GAAATGAAAATGTTGTCTGGACAAGCA
	VIC probe	TTCCCAGCACCTTC
	FAM probe	TTCCCAGAACCTTC
MDR-1 3435C>T	Forward primer	ATGTATGTTGGCCTCCTTTGCT
	Reverse primer	GCCGGGTGGTGTCACA
	VIC probe	CCCTCACGATCTCTT
	FAM probe	CCCTCACAATCTCTT

The positions of the SNPs are underlined

Statistical analysis

Single nucleotide polymorphisms were determined in 46 patients who received 800 mg imatinib daily and with available peripheral blood or bone marrow samples. Clinical end points of interest were cumulative incidence of MMR and CMR. The Hardy-Weinberg equilibrium test was performed for each of the SNPs. The cumulative incidences of MMR and CMR were calculated using competing risk analysis. Competing risks were disease progression to advanced phase CML, discontinuation of treatment before achieving response, or death without previous response. As an allogeneic stem cell transplantation (allo-SCT) was allowed as off protocol treatment if no cytogenetic response was acquired within 12 months or according to the physician's preference, those patients were censored at the date of allo-SCT. Cox regression was used to compare MMR and CMR between the allelic variants of each SNP. Analyses were performed without and with adjustment for Sokal risk score and dose of cytarabine (200 mg/m^2 vs 1000 mg/m^2). Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were determined. Kaplan-Meier curves were constructed to illustrate MMR and CMR. All reported P-values are two-sided.

Results

Patients

Baseline characteristics including sex, age, Sokal risk group, and daily dose of cytarabine (per course) of 46 patients included are shown in Table 2 and presented according to MDR1-genotype. All patients participated in the HOVON-51 study and received 800 mg imatinib daily. The median age at diagnosis was 49 years (range 30-65). No significant differences in age were apparent among the different MDR1 genotypes. Classification by Sokal risk score showed 16 low-risk patients (35%), 16 intermediate-risk patients (35%), and 12 high-risk patients (26%). Fifty percent of patients homozygous for allele 1236T, 2677T, and 3435T were classified as high risk according to Sokal, but these percentages did not differ significantly from the other genotypes. In addition to a daily dose of 800 mg imatinib, patients also received 2 courses of cytarabine at a dose of 200 mg/m^2 in 32 patients (70%) or 1000 mg/m^2 in 14 patients (30%). Also the dose of cytarabine received was distributed evenly among the different genotypes. Collectively, these results show that the baseline characteristics of the patients were not significantly different between the allelic variant for each polymorphism. The distribution of the allelic variants of the 3 SNPs, C1236T, G2677T/A, and C3435T, is shown in Table 3. Each of the 3 SNPs were in Hardy-Weinberg equilibrium. However, each combination of two SNPs was in strong linkage disequilibrium ($P<0.001$), as illustrated by the large number of patients with the same variant for each combination of two SNPs (bold face numbers). For example, 10 out of 12 patients homozygous for 1236C were also homozygous for 2677G and 9 of them were also homozygous for 3435C.

Table 2. *Patient characteristics of 46 genotyped CML patients [who received 800 mg imatinib daily]*

Allelic variant	No. (%)	Sex [No. (%)]		Age [median (range)]	Sokal risk group [No. (%)]				Daily dose Ara-C [No. (%)]	
		Male	Female		< 0.8	0.8-1.2	> 1.2	n.a.	200 mg/m²	1000 mg/m²
All patients	46	24 (52)	22 (48)	49 (30-65)	16 (35)	16 (35)	12 (26)	2 (4)	32 (70)	14 (30)
C1236T, n=43										
CC	12 (28)	8 (67)	4 (33)	48 (37-63)	6 (50)	3 (25)	2 (17)	1 (8)	8 (67)	4 (33)
CT	23 (53)	12 (52)	11 (48)	54 (30-65)	8 (35)	10 (43)	4 (17)	1 (4)	16 (70)	7 (30)
TT	8 (19)	4 (50)	4 (50)	40 (31-52)	2 (25)	2 (25)	4 (50)	-	6 (75)	2 (25)
G2677T, n=41										
GG	10 (24)	6 (60)	4 (40)	53 (37-63)	5 (50)	2 (20)	2 (20)	1 (10)	6 (60)	4 (40)
GT *	23 (56)	13 (57)	10 (43)	52 (30-65)	8 (35)	10 (43)	4 (17)	1 (4)	16 (70)	7 (30)
TT †	8 (20)	3 (38)	5 (63)	40 (31-52)	2 (25)	2 (25)	4 (50)	-	7 (88)	1 (13)
C3435T, n=44										
CC	10 (23)	6 (60)	4 (40)	50 (37-63)	5 (50)	2 (20)	2 (20)	1 (10)	5 (50)	5 (50)
CT	24 (55)	15 (63)	9 (38)	51 (30-65)	10 (42)	10 (42)	3 (13)	1 (4)	17 (71)	7 (29)
TT	10 (23)	3 (30)	7 (70)	40 (31-56)	1 (10)	4 (40)	5 (50)	-	8 (80)	2 (20)

n.a. indicates not available, and n, number of patients with data available for a specific SNP
* Includes 1 patient with GA
† Includes 1 patient with AT

Table 3. *Distribution of the allelic variants of C1236T, G2677T and C3435T*

Each cell contains the number of patients with a specific combination of two nucleotides. The numbers between brackets denote column percentages, based on patients with data on both SNPs available.

Allelic Variant	G2677T					C3435T			
	No.	GG	GT	TT	n.a.	CC	CT	TT	n.a.
All patients	46	10	23	8	5	10	24	10	2
C1236T									
CC	12	**10 (100)**	2 (9)	-	-	**9 (90)**	2 (8)	1 (11)	-
CT	23	-	**20 (87)**	1 (13)	2	-	**21 (88)**	2 (22)	-
TT	8	-	1 (4)	**7 (88)**	-	1 (10)	1 (4)	**6 (67)**	-
n.a.	3	-	-	-	3	-	-	1	2
G2677T									
GG						**9 (90)**	1 (5)	-	--
GT						1 (10)	**19 (86)**	3 (33)	-
TT						-	2 (9)	**6 (67)**	-
n.a.						-	2	1	2

n.a. indicates not available

Table 4. *Cumulative incidence of molecular response, by genotype*

		Major molecular response (MMR)					Complete molecular response (CMR)				
		At 1 year	Univariate		Adjusted †		At 1 year	Univariate		Adjusted †	
Allelic variant	No. (%)	% (s.e.)	HR (95% CI)	P *	HR (95% CI)	P *	% (s.e.)	HR (95% CI)	P *	HR (95% CI)	P *
All patients	46	59 (7)					22 (6)				
C1236T, n=43											
CC	12 (28)	92 (8)	1	.02	1	.03	42 (14)	1	.06	1	.01
CT	23 (53)	52 (10)	.32 (.14-.71)		.31 (.13-.71)		13 (7)	.36 (.16-.82)		.25 (.10-.63)	
TT	8 (19)	50 (18)	.33 (.12-.89)		.40 (.14-1.15)		25 (15)	.48 (.17-1.39)		.27 (.08-.97)	
G2677T, n=41											
GG	10 (24)	90 (9)	1	.11	1	.21	40 (15)	1	.13	1	.05
GT	23 (56)	61 (10)	.45 (.20-1.05)		.49 (.20-1.16)		22 (9)	.46 (.20-1.08)		.33 (.13-.85)	
TT	8 (20)	38 (17)	.36 (.13-.99)		.42 (.15-1.21)		13 (12)	.35 (.11-1.13)		.23 (.06-.88)	
C3435T, n=44											
CC	10 (23)	90 (9)	1	.04	1	.06	50 (16)	1	.10	1	.04
CT	24 (55)	58 (10)	.37 (.16-.83)		.35 (.15-.82)		17 (8)	.39 (.17-.92)		.33 (.13-.81)	
TT	10 (23)	40 (15)	.31 (.12-.83)		.35 (.12-.98)		10 (9)	.39 (.13-1.17)		.24 (.07-.83)	

s.e. indicates standard error; HR, hazard ratio; and CI, confidence interval

* *P* values are for the comparison of the molecular response rate between the different allelic variants of each genotype

† adjusted for Sokal risk group and dose Ara-C

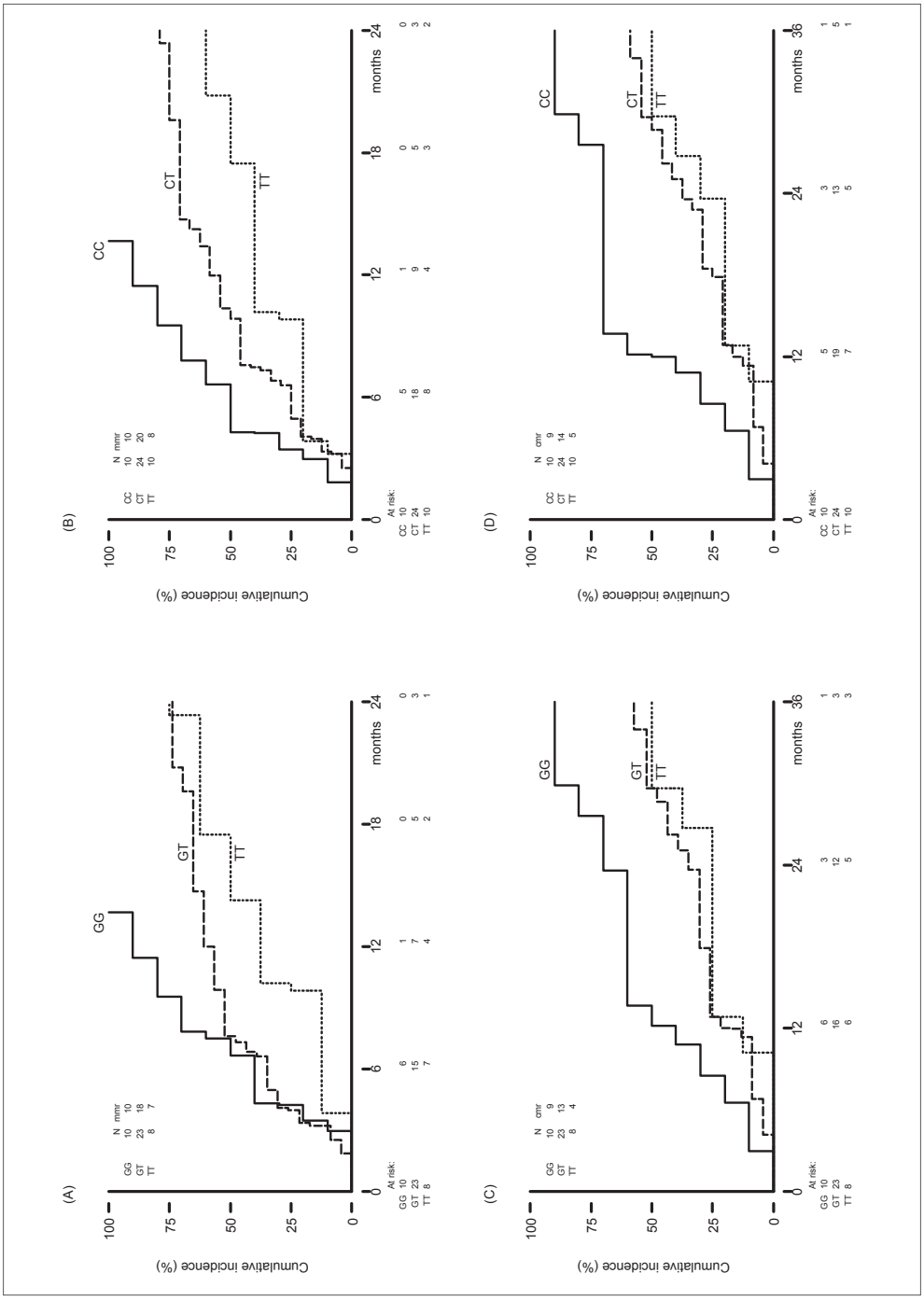

Figure 1. *Cumulative incidence of molecular response, by genotype*

(A) MMR, by G2677T (B) MMR, by C3435T (C) CMR, by G2677T (D) CMR, by C3435T

Molecular responses

Cumulative incidences of MMR and CMR by MDR1 genotype are presented in Table 4 and Figure 1. These responses were observed after a median follow-up of 46 months (range, 32-60 months). The overall incidences of a MMR and CMR were, respectively, 78% and 41% at 2 years from diagnosis. Molecular responses proved to depend strongly on SNP genotype. A cumulative incidence of MMR of 52% and 50% was observed in patient with genotype 1236CT or TT as compared to 92% in patients homozygous for 1236C (hazard ratio's: 0.32 and 0.33, P =.02; Table 4) in univariate analysis.The adverse outcome of MMR in patients heterozygous or homozygous for the 1236T allele remained statistically significant in a multivariate analysis with adjustment for Sokal risk group and dose of cytarabine. Hazard ratios to achieve a CMR for patients harboring the CT and TT alleles were in multivariate analysis, respectively, 0.25 (0.10-0.63) and 0.27 (0.08-0.97), P=.01, indicating a 4-fold reduction of the probability to obtain a CMR. Furthermore, also patients homozygous for allele 3435T showed a significant lower probability to obtain a MMR and CMR in multivariate analyses (Table 4, Figure 1). At 36 months from treatment, a total of 14 patients were considered resistant at the molecular level as a result of failing to achieve a CMR, including 4 patients with the 2677TT genotype and 1 patient with the 2677GG genotype.

Discussion

While most CML patients with a complete cytogenetic response (CCR) to first-line imatinib proceed to a MMR, only few patients develop a true complete molecular response (CMR). Major molecular responses have been reported to vary between 50-70% of upfront treated patients and complete molecular responses may occur in 10-40% of patients treated long-term with imatinib at a standard-dose[3,,27,28] Increasing the dose of imatinib has been suggested to be associated with higher molecular responses[29] but mature data from randomized studies are still lacking. The Dutch-Belgian HOVON cooperative group performed a dose escalating study exploring the combination of escalated imatinib with 2 short courses of iv cytarabine. Upfront combination appeared well tolerated[23] and mature efficacy data in patients receiving imatinib maintenance showed a relatively high percentage of molecular responses. Despite these favourable results, still approximately 40% of patients were considered molecular resistant, because of persistent molecular disease after 36 months of treatment. Persistent minimal (molecular) residual disease has been suggested to be accounted for by residual quiescent malignant stem cells, that are relatively insensitive to imatinib.[8,9] However, the precise mechanism of such resistance has remained elusive. Several possible explanations have been suggested, including enhanced drug efflux by P-gp, which is known to be relatively highly expressed by hematopoietic progenitor cells.[30,31] Here we addressed the question whether polymorphisms is the ABCB1 gene would be associated

with molecular response and resistance. It is shown that the molecular response in CML patients receiving high-dose imatinib strongly depends on SNP-genotype, which remained significant in multivariate analysis. Hazard ratio's to develop a CMR by TT homozygous patients of all 3 SNPs studied were in the range of 0.24 to 0.27, indicating a 4-fold lower probability to develop a CMR in these patients. Given the strong linkage equilibrium demonstrated by us and others[32] for these SNPs, it is not directly clear which individual SNP may account for the failure to develop a CMR.

Functional studies with a number of drugs addressing the role of these SNPs were performed by many groups and reviewed by Lepper et al.[33] A large number of clinical and preclinical studies were critically reviewed, largely showing inconsistent results, which may relate to the many drugs evaluated, different patient groups, and ethnic backgrounds among other possible explanations.[33] Whereas the C3435T and C1236T SNPs do not lead to an amino acid substitution, an altered function may result from changes in expression of the encoded protein, which may be explained by altered mRNA stability in case of the C3435T SNP.[19] Some studies suggested lower P-gp expression with the CC genotype in hematopoietic progenitor cells, whereas other studies showed a lower P-gp expression associated with the TT genotype[34,35] Functional studies in patients have shown both higher and lower clearances associated with the 3435TT genotype, but an elegant recent study using patient-samples and transfected cells more strongly suggested reduced mRNA stability and reduced expression by the CC genotype.[19] Recently, Gurney et al examined differences in day 1 and steady-state imatinib clearances according to SNP-genotype in patients receiving imatinib.[11] Less dose reduction and enhanced clearance appeared significantly associated with the TT genotype. Again, due to the strong linkage disequilibrium, that effect could not be attributed to a single SNP. Our results of a reduced molecular response in patients harboring the TT genotype would be compatible with their findings. Of note, the 2677TT genotype, which does lead to an amino acid substitution, has been associated with increased transport activity of P-gp before.[36] Collectively, these findings may provide an explanation for our findings and more specifically seem to suggest that enhanced clearance of imatinib by the 2677TT genotype in particular could be responsible for the significantly lower molecular response in our group of patients.

Only very few clinical studies so far have addressed the role of P-gp and it's SNPs in CML patients. Galimberti et al observed an inverse correlation between *BCR-ABL* copy number and MDR1 expression, possibly suggesting a role for MDR1 in clinical resistance.[37] While Gurney et al. studied imatinib clearance rates according to SNP-genotype as outlined above, Dulucq et al. addressed the question whether SNP-genotype would be associated with molecular response.[11,12] Although a positive correlation was observed, their findings rather suggested a better response in patients with the TT genotype, which strongly contrasts with our findings. Several explanations may be brought forward. First, pharmacokinetic resistance rather than tumor cell resistance could account for these differences, as the responses observed by

Dulucq et al. were strongly related to imatinib plasma levels. Their study included patients treated with 400-600 mg of imatinib, which may yield a wider range of plasma levels as compared to patients treated with a fixed dose of 800 mg imatinib.[6,7] In addition, the higher dose of imatinib has been suggested as a strategy to circumvent pharmacokinetic resistance by exceeding the level of 1,000 ng/ml.[38] Furthermore, the inclusion of both chronic phase and accelerated phase patients may not allow to focus selectively on residual molecular disease, as only very few accelerated phase patients can be expected to develop a molecular response within 12 months. We included only first chronic phase CML-patients receiving high-dose imatinib in order to circumvent pharmacokinetic resistance as much as possible and focus on tumor cell resistance. Thereby, our intention was to evaluate long-term (>24 months of therapy) molecular responses and primary molecular resistance in patients already showing favourable cytogenetic responses. Although numerous differences between our study and the French study preclude a direct comparison, results from the latter study may seem to suggest that pharmacokinetic resistance, which may be accounted for by MDR1, but also by a number of other mechanisms, plays a more important role in patients receiving 400 mg imatinib.

In conclusion, molecular resistance in CML patients receiving high-dose imatinib appeared strongly associated with ABCB1 gene SNPs, suggesting a role for P-gp mediated drug efflux in malignant hematopoietic progenitor cells, that may possibly account for persistent molecular residual disease in patients favourably responding to imatinib.

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6

DISCRIMINATION OF THE LEUKEMIC AND NORMAL STEM CELL IN THE BONE MARROW OF PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Abstract

Inherent tyrosine kinase inhibitor insensitivity of CML hematopoietic stem cells prevents eradication of the disease by these drugs and is probably involved in development of tyrosine kinase inhibitor resistance. To improve treatment results, more knowledge about CML stem cells is therefore needed. Previously, these cells could only be identified indirectly by using culture techniques. We now present a new flowcytometric approach to directly distinguish CML stem cells from their normal counterparts within single patient samples. In 24 newly diagnosed CML patients CML $CD34^{+}CD38^{-}$ stem cells could be discriminated from normal stem cells by higher CD34 and CD45 expression and different forward/sideward light scatter properties. In addition, expression of CD7, CD11b and CD56 helped to distinguish malignant from benign stem cells. FISH analysis on FACS sorted cells proved that populations were *BCR-ABL* positive or negative and long term liquid culture assays with subsequent CFU assays and FISH analysis proved their stem cell character. Patients with a large proportion of non-leukemic stem cells had significantly lower clinical risk scores (Sokal, Euro), than patients with few remaining normal stem cells. This new technique will expand our possibilities to identify new CML stem cell specific targets and may improve efficacy assessment of CML treatment.

Introduction

Chronic myeloid leukemia (CML) is a clonal disease that originates from a transformed hematopoietic stem cell or multipotent progenitor cell.[1,2] It is characterized by the presence of the Philadelphia translocation t(9;22) encoding for a new fusion gene (*BCR-ABL*) with constitutive tyrosine kinase activity. The BCR-ABL tyrosine kinase activity leads to the accumulation of both mature and immature myeloid cells in bone marrow, peripheral blood and spleen through increased cycling of CML stem cells. This results in an enlarged colony forming compartment of Ph+ progenitor cells that in itself possess near-normal CFC frequency.[3] When treated inadequately, the expanding leukemic stem cell and progenitor pool increasingly suppresses residual normal hematopoiesis and eventually, due to its BCR-ABL-induced genetic instability[4], progressively acquires secondary genetic abnormalities, which ultimately leads to transformation of the disease into a rapidly fatal blast crisis.

Fortunately, major breakthroughs in CML research have led to a revolution in therapy by the introduction of a specific inhibitor of BCR-ABL activity, imatinib and, more recently, to even more potent tyrosine kinase inhibitors like nilotinib and dasatinib. These drugs can induce complete hematologic responses in virtually all patients, complete cytogenetic responses in almost 90% and progression-free survival in 84% of patients.[5-7] In a large clinical trial, patients who attained a reduction of the *BCR-ABL* transcript level that was more than 1000-fold lower (major molecular response) than that at diagnosis had durable responses without any event of progression in a large prospective clinical trial.[5]

Unfortunately, not all patients attain such a favourable response. Primary or secondary resistance, defined at the hematologic level, may develop in approximately 4% of chronic phase cases, while cytogenetic resistance may be observed in up to 10-15% of patients.[5,8] Besides, a small number of patients presents in advanced phases of the disease, where the efficacy of imatinib and, to a lesser extent also that of nilotinib and dasatinib, is limited. Most important, tyrosine kinase inhibitor therapy cannot eradicate the disease, because leukemic stem cells appear to be inherently resistant against these compounds. This probably relates to several mechanisms. *Firstly*, a strong increase in *BCR-ABL* mRNA- and protein expression (up to 100-fold and 3-10 fold, respectively) was found in the most primitive stem cell compartment compared to more committed progenitor cells.[9-11] This may lead to insuffcient inhibition of its kinase activity by imatinib and thereby to reduced cell killing by the drug. The high BCR-ABL expression parallels the autocrine production of interleukin-3 and GMCSF which is only present in the most primitive stem cells.[11] Possibly, this autocrine loop offers additional protection of stem cells against imatinib. *Secondly*, imatinib influx into the most primitive stem cells is hampered by their very low expression of Oct-1, which pumps imatinib into the cell.[12,13] *Thirdly*, the mRNA levels of the ABCB1 (also known as P-glycoprotein) and ABCG2 (also called BCRP) efflux pumps for which imatinib is a substrate, were 2-fold higher in the most primitive cells compared to more mature progenitors.[14,15]

This may explain low sensitivity of primitive stem cells to both imatinib as well as to conventional chemotherapeutic drugs.

For development of more effective strategies that are able to eliminate these CML stem cells, better insight in their functional properties is needed. In vitro long term culture experiments and fluorescent in situ hybridization (FISH) analysis or RT-PCR have revealed that both BCR-ABL positive and negative stem cells co-exist in the $CD34^+CD38^-$ stem cell compartment in CML, however, up to now no information has become available on how to study these separately in a direct way in freshly obtained diagnosis samples.

Recently, we were able to demonstrate that malignant and benign stem cells in AML can be discriminated in one sample by using flowcytometry.[16,17] Using a similar approach, we now show for the first time that malignant and benign stem cells in CML patients can also be distinguished on the basis of light scatter properties, CD34 and CD45 expression and hematopoietic lineage marker expression. This new finding opens the way to facilitate stem cell specific target discovery and allows to assess the number of residual malignant stem cells under treatment with tyrosine kinase inhibitors, which may be an important parameter for the evaluation of their therapeutic efficacy.

Patients, materials and methods

Samples from patients and healthy volunteers; cell preparation

After informed consent, peripheral blood and bone marrow were obtained from newly diagnosed, untreated CML patients. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-paque (1.077 g/ml; Amersham Biosciences, Freiburg, Germany) followed by a red cell lysis step with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 10 minutes at 4°C, or by direct lysis only. For control experiments, bone marrow samples from eight healthy volunteers were used.

CD34+ cell selection

Ficoll-separated mononuclear cells were incubated in PBS containing 5 mM EDTA and 0.5% human serum albumin (HSA) for 30 minutes at room temperature with CD34 Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Cells were washed twice in PBS/5mM EDTA/0.5% HSA and then selected using the AutoMacs automated selection device using its Possel-DS mode (Miltenyi Biotec). Purity was checked using flow cytometry and was always more than 95% $CD34^+$. In several cases, long term clonogenic assays on sorted populations were done on thawed CD34+ cells that had been cryopreserved in RPMI/20% FCS/10% DMSO.

Fluorescence-activated cell sorting

Fresh isolated mononuclear cells or purified CD34+ cells were incubated with monoclonal antibodies for 15 minutes at room temperature, washed once in PBS containing 0.1% HSA and analyzed by flow cytometry. Anti-CD34 phycoerythrin-Cy7 (PE-Cy7), anti-CD45 fluorescein isothiocyanate (FITC), anti-CD38 allophycocyanin (APC), anti-CD7-phycoerythrin (PE), anti-CD11b-PE, anti-CD19-PE, anti-CD22-PE, anti-CD56-PE and Via-Probe (7-amino-actinomycin D, 7AAD) were all from BDBiosciences (Erembodegem, Belgium). Anti-CD2-PE and anti-CD5-PE were from DAKO (Heverlee, Belgium). Anti–CLL-1-PE and anti-DiNitroPhenol (DNP) were from Crucell (Leiden, the Netherlands). Every test tube contained anti-CD45-FITC, anti-CD34-PE-Cy7, anti-CD38-APC, 7-AAD and one of the PE-labeled antibodies. IgG1-PE was used as a control for PE labeled anti-CD2, -CD5, -CD19, -CD56 and -CD7, IgG2a-PE was used for PE-labeled anti-CD11b and IgG2b for CD22-PE. For anti-CLL1, DNP-PE was used as a control. PBS was used as a control for anti-CD34-PE-Cy7, CD38-APC and -CD45-FITC. 7-AAD was always included to gate out apoptotic/dead cells before stem-cell assessment. Data acquisition was performed using a 3-laser FACSCanto II flowcytometer (BD Biosciences) and analysis was performed using FACSDiva software (BD Biosciences).

FACS gating strategy and FACS sorting

Using five-color fluorescence-activated cell sorting (FACS) analysis, light scatter properties and expression of lineage marker (CD2, CD5, CD7, CD11b, CD19, CD22 and CD56) and CLL-1 were studied. These single markers are absent on normal $CD34^{+}CD38^{-}$ cells.[16] $CD34^{+}$ blasts were identified in a population characterized by dim expression of CD45, low sideward scatter (SSC) and 7-AAD negativity. Within this $CD34^{+}/CD45^{dim}/SSC^{low}/7AAD^{-}$ population, the $CD34^{+}CD38^{-}$ compartment was defined with the use of a PBS control. Next, these $CD34^{+}38^{-}$ cells were backgated into the FSC/SSC plot, into a CD34/CD45 plot and into a lineage marker/FSC or lineage marker/CD45 plot. In the AML samples with $CD34^{+}$ blasts that were used as controls for light scatter properties, malignant $CD34^{+}38^{-}$ cells were discriminated from their normal counterparts on the basis of their aberrant lineage marker expression. Marker positive cells in the $CD45^{dim}/SSC^{low}$ compartment of $CD34^{-}$ AML were also considered malignant.

Using the above-mentioned gating strategy, cells were sorted on a FACSAria cell sorter (BD) directly onto glass slides, or into PBS/0.1% HSA in case further culture experiments were planned.

Colony forming unit (CFU)- and long term suspension culture assays

For CFU assays, cells obtained after CD34 isolation and FACS sorting of CD34+ subpopulations, were cultured in semisolid medium containing a-methylcellulose (Methocult GF H4434; StemCell Technologies, Vancouver, Canada) at 37°C in 5% CO_2 in a humidified incubator. The number of colonies was counted after 14 days culture. Colonies of different sizes were picked using very thin hand made glass pipettes or a standard laboratory pipette (Rainin, Mettler-Toledo, Tiel, The Netherlands) with 10 ul pipette tips and spread onto glass slides for FISH analysis (see below).

Long term suspension cultures were performed essentially as has been previously described.[18] The sorted (sub)populations were suspended in CellGro medium (Cellgenix, Vancouver, Canada) containing 20 ng/ml IL-3, 100 ng/ml Flt-3 ligand and 100 ng/ml SCF (all from Pepro Tech, Basel, Switzerland) prior to plating in 96 round-bottom- or 24-wells plates, depending on the number of available cells (Greiner, Frickenhausen, Germany). Suspension cultures were incubated at 37°C in 5% CO_2 and received weekly half-medium and -cells changes (demipopulation). If sufficient cells (>20.000) were available, CFU-assays were performed every week. This weekly procedure was continued until no CFU output was present anymore. When sufficient cells were available, cultures were maintained and weekly CFU-assays were continued for up to eight weeks. In cases where the number of cells was very low, we chose to harvest all cells at one time point only, i.e. 5 weeks. Subsequently, all harvested cells were cultured in a CFU assay.

Fluorescent in situ hybridization (FISH)

For FISH analysis, slides were dehydrated in ethanol 70, 96 and 100% for 5 min at RT, fixed in methanol: acetic acid (3:1) and stored at -20°C until use. After thawing, slides were incubated for 90 min at 37°C in 100 mg RNAse A (Boehringer Mannheim, Germany)/ml 2x standard saline citrate (SSC) and washed three times for 2 min in 2xSSC at 37°C. Next, slides were incubated for 10 min at 37°C in 0.01% pepsin (Sigma-Aldrich, St Louis, MO, USA) in 10mM HCl, and washed two times for 5 min in PBS at room temperature (RT). Afterwards, the slides were incubated for 10 min in 3.7% formaldehyde in PBS at RT and washed two times for 5 min in PBS. Denaturation in 70% formamide was performed at 72°C, for 5 min. Next, dehydration was performed two times for 5 min in 70% EtOH at -20°C, 5 min in 96% EtOH at RT and finally 5 min in 100% EtOH at RT. Slides were air-dried. Hybridization with the Vysis® LSI® BCR/ABL ES Dual Color Translocation Probe (Abbott BV, Hoofddorp, The Netherlands) took place overnight under moist conditions at 37°C. Afterwards, slides were washed in 50% formamide/2xSSC at 42°C three times for 10 min. Next, slides were washed at 42°C, for 10 min in 2xSSC, washed in 2xSSC/0.05% Tween at 42°C for 5 min and finally washed in 1xPBS at RT for 4 min. Slides were incubated for 9 min in DAPI

(Sigma-Aldrich) /PBS (0.125 ug/ml) at RT. After this, slides were dehydrated in ethanol 70, 96 and 100% for 1 min at RT and air-dried. Vectashield (Vector Laboratories, Brunschwig Chemie, Amsterdam, The Netherlands) was applied to the slides before analysis on a Zeiss fluorescence microscope. Colonies had to consist of at least ten cells and were scored BCR-ABL positive or negative if at least 90% of all cells from that colony were either BCR-ABL positive or –negative. The background level of false positives for this probe-set is around 10%. To prevent bias, scoring was performed in a blinded fashion.

Statistical analysis

Expression of lineage markers is given in arbitrary fluorescence units as generated by the FACS Diva software. As FACS Diva software may generate mean fluorescence values of marker-negative populations that can have a value near or below zero, we chose to calculate fluorescence ratios by dividing the median fluorescence of the positive population by the fluorescence of the negative control at the level that excludes the upper 2.5% of events. Typical values for negative populations are below 0.35. Fluorescence ratios were compared using the Mann-Whitney U-test for non-parametric variables (SPSS 17.0 software package, SPSS, Chicago, IL). Sokal and Euro-scores were calculated according to Hehlmann et al[19] and also compared using the Mann-Whitney U-test for non-parametric variables. *P* values less than .05 were considered to indicate a statistically significant difference.

Results

We set out to investigate whether the previously demonstrated marker and light scatter differences between leukemic and normal stem cells in AML could also be applied to CML.[16,17] To this end, we examined fresh bone marrow and/or peripheral blood samples of 24 newly diagnosed CML patients by flowcytometry. Patient characteristics are shown in Table 1. Figure 1A depicts the gating strategy to define the total $CD34^+/CD38^-$ stem cell compartment. Cells were further backgated into a forward scatter/sideward scatter plot (FSC, reflecting cell size; SSC, reflecting cell granularity), FSC/lineage marker plot and CD34/CD45 plot. Using this approach, we identified three different groups of patients (indicated as I, II, III), ultimately differing in the degree to which CML and normal stem cells could be discriminated.

Table 1. *Patient characteristics.*

Group I: patient numbers 1-9; group II: 10-19; group III: 20-24

Patient number	Age (yr)	M/F	Sokal score	Euro score	Spleen size (cm BCM)	Leukocyte count (x10^9/l)	Cytogenetics	Breakpoint
1	74	M	0.84	1036	0	40.2	100% Ph$^+$, del 9q34	b3a2
2	53	F	0.75	1665	0	38.9	100% Ph$^+$	b3a2/b2a2
3	67	M	0.94	919	6	182	100% Ph$^+$, 13% also -8	b3a2
4	55	F	0.83	1215	0	44.2	100% Ph$^+$	unknown
5	43	F	0.6	41	0	44.6	nd (*BCR-ABL*$^+$)	b3a2/b2a2
6	33	M	0.77	776	5	73	100% Ph$^+$	b2a2
7	56	F	0.85	1274	0	71.4	97% Ph$^+$	b3a2
8	66	F	0.78	1320	0	45.9	100% Ph$^+$	b3a2/b2a2
9	42	M	na[1)]	na[1)]	na[1)]	366	100% Ph$^+$	b3a2
10	68	F	1.13	1798	0	101	100% Ph+	b3a2/b2a2
11	46	M	1.44	2143	12	421	84% Ph$^+$ [2)]	b3a2
12	64	M	1.53	2883	5	282	81% Ph$^+$ [2)]	b3a2
13	60	M	1.1	1052	5	500	100% Ph$^+$	b3a2
14	75	F	1.26	1380	5	245	100% Ph$^+$	b3a2/b2a2
15	59	M	1.01	1359	3	204	100% Ph$^+$	b2a2
16	19	M	0.51	449	0	19.9	97% Ph$^+$	b2a2
17	38	M	0.85	1334	8	141	93% Ph$^+$ [2)]	b3a2
18	47	M	na	na	na	176	92% Ph$^+$ [2)]	b2a2
19	44	F	0.75	753	0	30.0	100% Ph$^+$	b2a2
20	65	M	2.38	3289	19	73.1	100% Ph$^+$	b3a2
21	67	M	2.47	4237	15	127	100% t(1;9;22;13)	b3a2
22	52	F	0.76	1378	0	207	91% Ph$^+$,del 9q34[2)]	b3a2
23	35	F	1.37	1699	15	316	100% Ph$^+$	b3a2
24	53	F	1.86	3732	11	260	100% Ph$^+$	b2a2

BCM: below costal margin
nd: not done
na: not available
[1)] Sokal and Euro-scores cannot be calculated, because spleen size could not be assessed
[2)] assessed by FISH only

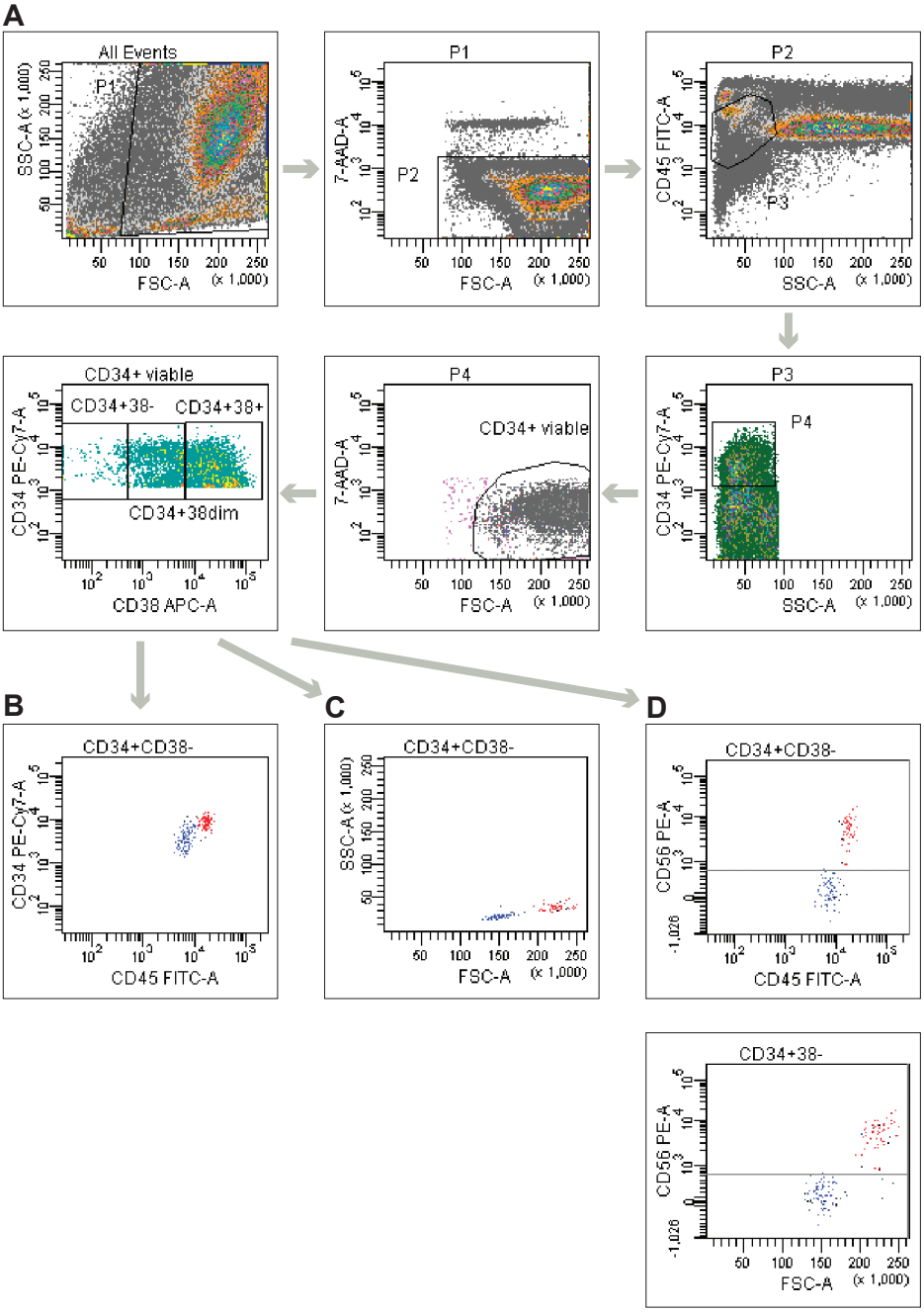


Figure 1. *Gating strategy to identify CD34⁺CD38⁻ stem cells in all CML patients and FSC/SSC characteristics and expression patterns of CD34, CD45 and lineage marker in a representative patient sample.*

Total number of sorted cells in this case (p1) 8x10⁵. The horizontal lines in Figure 1D represents the maximum level of the control.

Group I.

Patterns of FSC/SSC and CD34/CD45 expression.

Figure 1 shows an example of this group (patient number 1 in Table 1). Two clearly different populations could be identified using the gating strategy shown in Figure 1B and C: of all events with high CD34 and CD45 expression, 95% were also high in FSC and SSC. In contrast, 99% of the cells with lower CD34/CD45 expression were FSC^{low}/SSC^{low}.

We found similar and very clear CD34/CD45 and FSC/SSC patterns in eight other cases (patient numbers 2-9), i.e. at least 90% of $CD34^{high}/CD45^{high}$ events were FSC^{high}/SSC^{high}, while at least 90% of $CD34^{low}/CD45^{low}$ events were FSC^{low}/SSC^{low}. For the whole group of nine patients in this group, the number of $CD34^{low}/CD45^{low}$ cells as percentage of the total number of viable $CD34^{+}$ cells (see Table 2) was almost two times that of the $CD34^{high}/CD45^{high}$ cells. Likewise, FSC^{low}/SSC^{low} cells outnumbered FSC^{high}/SSC^{low} cells in this group twofold.

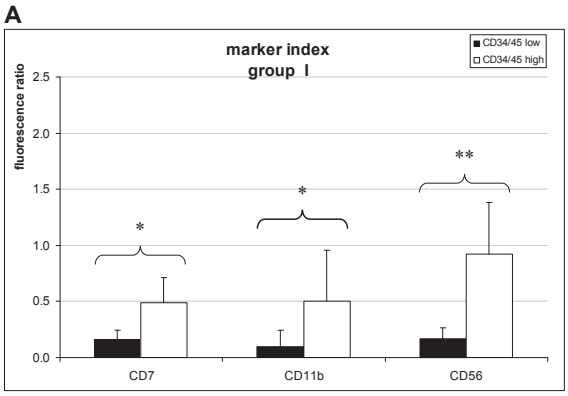
Table 2. *CML stem cell populations defined by CD34 and CD45 expression.*

$CD34^{high}/45^{high}$ and $CD34^{low}/45^{low}$ cells are presented as a percentage of total number of viable CD34+ cells in group I (n=9), group II (n=10) and group III (n=5).

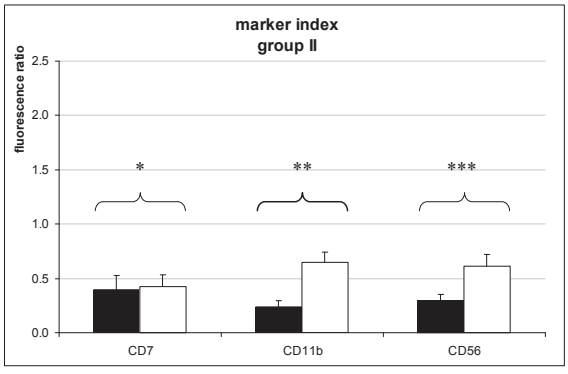
Patient group	No. of patients	$CD34^{low}/CD45^{low}$ percentages (median, range)	$CD34^{high}/CD45^{high}$ percentages (median, range)	ratio $CD34^{low}/CD45^{low}$ to $CD34^{high}/CD45^{high}$	FSC^{low}/SSC^{low} percentages (median, range)	FSC^{high}/SSC^{high} percentages (median, range)	ratio FSC^{low}/SSC^{low} to FSC^{high}/SSC^{high}
I	9	0.32 (0.04-1.32)	0.17 (0.01-1.45)	1.89	0.31 (0.02-1.44)	0.16 (0.03-1.44)	1.95
II	10	0.19 (0.06-0.40)	0.58 (0.09-6.97)	0.34	0.16 (0.04-2.22)	0.54 (0.11-4.86)	0.29
III	5	0.04 (0.01-0.14)	0.67 (0.03-2.26)	0.05	0.01 (0.004-0.07)	0.56 (0.04-1.37)	0.02

Lineage marker expression

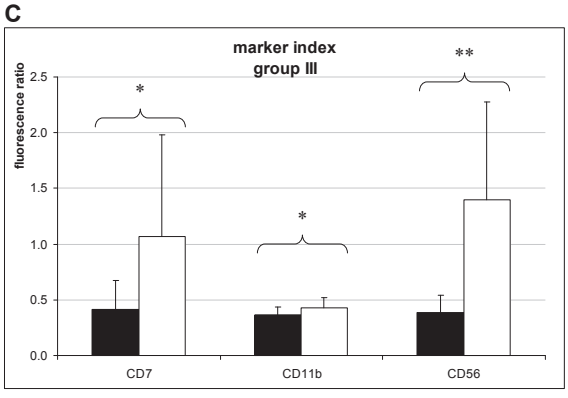
An example of lineage marker expression is shown in Figure 1D: 96% of the $CD34^{high}/CD45^{high}$ cells were CD56 *positive*, while the $CD34^{low}/CD45^{low}$ cells were marker *negative* for 96%. In agreement with this, lineage marker (CD7, CD11b and CD56) expression in an additional group of eight patients was always negative in the $CD34^{low}/CD45^{low}$ population, but (partially) positive in the $CD34^{high}/CD45^{high}$ population. Figure 2A summarizes marker expression for all nine patients. Overall, CD56 was the marker that most clearly differed in expression between $CD34^{low}/CD45^{low}$ and $CD34^{high}/CD45^{high}$ cells. No aberrant expression at all was seen for a set of markers important for AML stem cell identification i.e. CD2, CD5, CD19, CD22 or CLL-1, neither in $CD34^{high}/CD45^{high}$, nor in $CD34^{low}/CD45^{low}$ cells (data not shown).



* : *P*>.1 ; ** : *P*=.07



* : *P*>.1 ; ** : *P*=.002 ; *** : *P*=.023



* : *P*>.1 ; ** : *P*=.076

Figure 2. *Mean lineage marker expression in CD34^{low}/CD45^{low} versus CD34^{high}/CD45^{high} in CML patients.*

Black bars: CD34^{low}/CD45^{low}, white bars: CD34^{high}/CD45^{high}. Typical values of negative populations are below 0.35, as defined in "Patients, materials and methods" section. Error bars represent SEM.

FISH analysis

FSC^{high}/SSC^{high} and FSC^{low}/SSC^{low} or CD34^{high}/CD45^{high} and CD34^{low}/CD45^{low} cells were sorted for FISH analysis in four samples. This proved that, similar to AML, the described parameters segregate normal from leukemic stem cells (see first part of Table 3).

Table 3. *BCR-ABL status in sorted presumed normal and CML stem cells.*

Cells were sorted directly onto glass slides and subjected to FISH as described in Patients, Materials and Methods.

Patient group	Patient number	*BCR-ABL* positivity (%)	
		CD34^{low}/45^{low} or FSC^{low}/SSC^{low}	CD34^{high}/45^{high} or FSC^{high}/SSC^{high}
I	1	5[a]	91[a]
	4	28[b]	100[b]
	5	6[a]	82[a]
	9	13[a]; 0[b]	98[a], 93[b]
II	10		89[b]
	12	34[b]	100[b]
	14		100[b]
	15	48[b]	
	19	35[a]	96[a]
III	20	29[c]	90[a], 100[b]
	21	81[b]	100[b]
	24	96[b]	95[b]

[a)] sorted on FSC/SSC
[b)] sorted on CD34/45
[c)] sorted on CD34^{low}/CD45^{low}/FSC^{low}/SSC^{low}

Stem cell cultures of CD34/CD45 and FSC/SSC defined populations

Sorted CD34^{high}/CD45^{high} or FSC^{high}/SSC^{high} cells were plated in long term cultures and clonogenic capacity was measured every week for at least five consecutive weeks as far as allowed by cell numbers. Quantitative analyses of the clonogenic capacity of the separate sorted populations are presented in Figure 3A, which shows that in most cases the subpopulations sorted on these characteristics have long-term clonogenic ability. The colonies were picked up and subjected to FISH analysis. Results are shown in Table 4 and prove that the colonies from the cultured sorted subpopulations represent CML and normal stem cells, respectively.

Summarizing, CML and normal stem cells can be clearly discriminated based on FSC/SSC characteristics and CD34/CD45 expression patterns and in some cases by aberrant lineage marker expression.

The described subcompartments in the remaining 15 patient samples were less well defined mostly due to larger differences in frequency between these. In the most extreme cases, one of the populations may be virtually absent, which not only hinders the separate identification of both populations, but may even prevent to assign the quality "normal" or "leukemic" to the high frequency population. In order to still enable separate recognition and qualification, we investigated whether lymphocytes might serve as an internal control for light scatter properties and CD45 levels of $CD34^{+}CD38^{-}$ cells. In addition, as external controls, we compared light scatter properties of $CD34^{+}38^{-}$ cells of normal bone marrow samples with lymphocytes in the same samples.

In the CML samples, the FSC^{low}/SSC^{low} stem cell population had a consistently higher FSC than the corresponding lymphocytes in the same sample. The ratio of both ("FSC ratio") was 1.09 (mean, range 1.00-1.21). In contrast, the mean FSC ratio of the FSC^{high}/SSC^{high} population was 1.58 (range 1.40-1.72) and thus showed no overlap with the FSC^{low}/SSC^{low} population. Although in control bone marrow the normal $CD34^{+}CD38^{-}$ stem cell population had a higher FSC ratio than the presumed normal FSC^{low}/SSC^{low} stem cell population in the CML samples, it was still lower than that of the FSC^{high}/SSC^{high} population in 17 of 23 tested CML samples. For CD45, the ratio between the $CD34^{low}/CD45^{low}$ and the lymphocytes population in the CML samples was 0.17 (range 0.13-0.23) and 0.34 (range 0.29-0.52) for the $CD34^{high}/CD45^{high}$ population, again showing no overlap between both populations. By using these light scatter and CD45 expression ratio's, the identity of the individual cell populations could now much better be determined in any sample, especially in those cases where only one population could be discerned. The remaining 15 patient samples were now analyzed accordingly and divided in two groups depending on the extent of discrepancy between CD34/CD45 expression and FSC/SSC properties.

Table 4. *FISH results of long term liquid culture derived colonies.*

Colonies were scored BCR-ABL positive if at least 10 cells were evaluable and at least 90% was positive. Patients 1, 2, 5 and 7 belong to group I, patients 12 and 13 to group II. n= number of colonies tested.

Patient group	Patient number	sorted populations			
		FSC^{low}/SSC^{low} or $CD34^{low}/CD45^{low}$		FSC^{high}/SSC^{high} or $CD34^{high}/CD45^{high}$	
		n	$BCR\text{-}ABL^{+}$	n	$BCR\text{-}ABL^{+}$
I	1[a]	4[c]	1	9	8
	2[b]	4	0	10	10
	5[a]	24	0	d	d
	7[b]	23	0	17	17
II	12[b]	18	7	26	26
	13[b]	[e]	e	21	13

[a] sorted on FSC/SSC
[b] sorted on CD34/CD45
[c] no growth at 5 weeks, data derived from 4 weeks colonies
[d] sort of FSC^{high}/SSC^{high} failed
[e] sort of $CD34^{low}/CD45^{low}$ failed

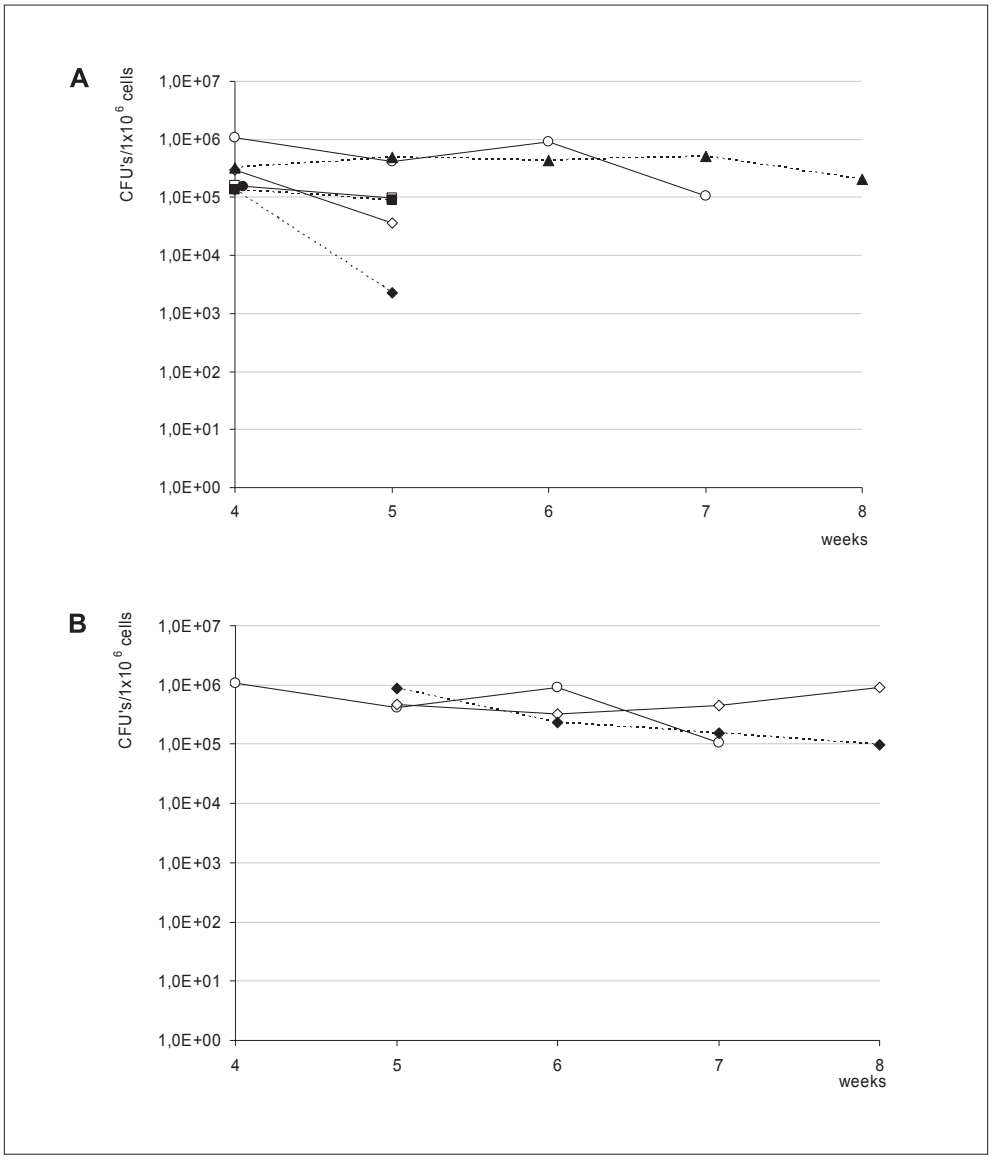


Figure 3. *Clonogenic capacity after long term culture of presumed leukemic ($CD34^{high}/CD45^{high}$ or FSC^{high}/SSC^{high}) and normal ($CD34^{low}/CD45^{low}$ or FSC^{low}/SSC^{low}) stem cells.*

On the x-axis, the number of weeks that cells had been in culture before plating in CFU-assay is given. Results of bone marrow and peripheral blood were averaged within patients. Open data points: $CD34^{high}/CD45^{high}$ or FSC^{high}/SSC^{high} cells, filled data points: $CD34^{low}/CD45^{low}$ or FSC^{low}/SSC^{low} cells.
A: Group I. Circles: patient no. 1, diamonds: patient no. 2, triangles: patient no. 5, squares: patient no. 7.
B: Group II. Circles: patient no. 11, diamonds: patient no. 12.

Group II.

Patterns of FSC/SSC and CD34/CD45 expression.

This group contains ten patients (patient numbers 10-19, see Table 1) in whom the $CD34^{high}/CD45^{high}$ population was still clearly identifiable, but in which the $CD34^{low}/CD45^{low}$ population was less well defined compared to group I patients. This seemed partly due to their much lower frequencies compared to the $CD34^{high}/CD45^{high}$ cells (see Table 2). For all ten patients in this group, $CD34^{low}/CD45^{low}$ cells as percentage of the total number of viable CD34+ cells was a factor three to four lower than that of the $CD34^{high}/CD45^{high}$ cells (see Table 2). In contrast to group I, in which at least 90% of $CD34^{low}/CD45^{low}$ were FSC^{low}/SSC^{low}, group II was defined to have between 10% and 50% FSC^{high}/SSC^{high} cells in the $CD34^{low}/CD45^{low}$ compartment. A representative example of this group is shown in Figure 4A.

Lineage marker expression

Lineage marker expression in the $CD34^{low}/CD45^{low}$ population in this group was virtually absent for CD11b and CD56, but low expression of CD7 was seen (fluorescence ratio 0.39, see Figure 2B). Compared to group I, for CD11b and CD56 the differences with the $CD34^{high}/CD45^{high}$ population were smaller compared to group I, but more significant, apparently due to less variability between patients. When we subdivide lineage marker expression in the three CD34/CD45 and FSC/SSC defined subpopulations ($CD34^{low}/CD45^{low}/FSC^{low}/SSC^{low}$, $CD34^{low}/CD45^{low}/FSC^{high}/SSC^{high}$ and $CD34^{high}/CD45^{high}/FSC^{high}/SSC^{high}$), only the two FSC^{high}/SSC^{high} populations had aberrant marker expression, together with FISH analysis (see next paragraph) strongly suggesting CML character (see Figure 5). Similar to group 1 patients there was no aberrant expression of CD2, CD5, CD19, CD22 or CLL-1 in any of the ten patient samples (data not shown).

FISH analysis

In three patients of this group, $CD34^{high}/CD45^{high}$ cells were sorted, which revealed on average 96% (range 89-100%) *BCR-ABL* positive interphases, while in two cases, sufficient $CD34^{low}/CD45^{low}$ cells were available for FISH analysis. In concordance with marker expression, these were partly *BCR-ABL* positive: 48 and 34%, respectively (Table 3, second part). An additional patient sample was sorted on FSC^{high}/SSC^{high} and $^{low/low}$ expression. FSC^{high}/SSC^{high} cells were 96% *BCR-ABL* positive, FSC^{low}/SSC^{low} cells were 35% *BCR-ABL* positive (see Table 3, second part). In retrospect, and after re-analysis of all cases, in this sample the upper limit of the FSC^{low}/SSC^{low} sorting gate turned out to include a small number of FSC^{high}/SSC^{high} events.

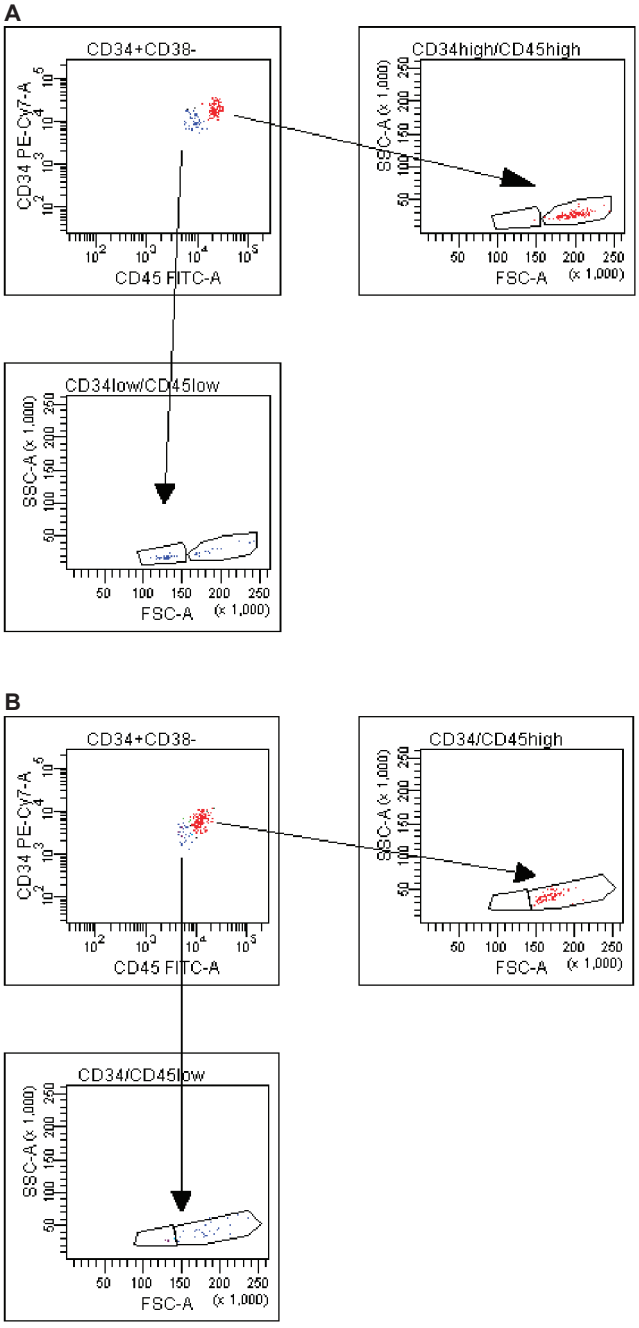


Figure 4. *FSC/SSC characteristics of CD34/CD45 defined subpopulations in patient groups II (4A) and III (4B).*

Gating as performed in Figure 1. Note the presence of both FSC^{low}/SSC^{low} cells and FSC^{high}/SSC^{high} cells within the $CD34^{low}/CD45^{low}$ compartment in group II, but also the almost complete absence of FSC^{low}/SSC^{low} cells within the $CD34^{low}/CD45^{low}$ compartment in group III.

Stem cell cultures of CD34/CD45 and FSC/SSC defined populations

Long term cultures in this group were done in two patients, based on CD34/CD45 expression. Both $CD34^{high}/CD45^{high}$ and $CD34^{low}/CD45^{low}$ cells showed stem cell activity. In line with the direct sort experiments, the $CD34^{low}/CD45^{low}$ compartment of patient no. 12 (Table 4) that consisted for a high percentage of FSC^{high}/SSC^{high} cells, contained a mixed population of *BCR-ABL* positive and *BCR-ABL* negative colonies, while all $CD34^{high}/CD45^{high}$ cells generated *BCR-ABL* positive colonies. In the second patient (no. 13) technical failure prevented long term culture of the $CD34^{low}/CD45^{low}$ cells. $CD34^{high}/CD45^{high}$ cells generated, in contrast to what was expected, colonies that were only 62% *BCR-ABL* positive. In retrospect, and after re-analysis of all cases, the gate for $CD34^{high}/CD45^{high}$ was set slightly too wide, resulting in admixture with some $CD34^{low}/CD45^{low}$ cells.

Thus, similar to the situation in group I, *BCR-ABL* negative stem cells are $CD34^{low}/CD45^{low}$, FSC^{low}/SSC^{low} and lineage marker negative. However, in contrast with group I, *BCR-ABL* positive cells, may be both $CD34^{high}/CD45^{high}$ and $CD34^{low}/CD45^{low}$. Irrespective of this, *BCR-ABL* positive cells were always FSC^{high}/SSC^{high} and may carry lineage markers. The FSC/SSC and, when present, the lineage marker properties thus offer the best discrimination between CML and normal stem cells.

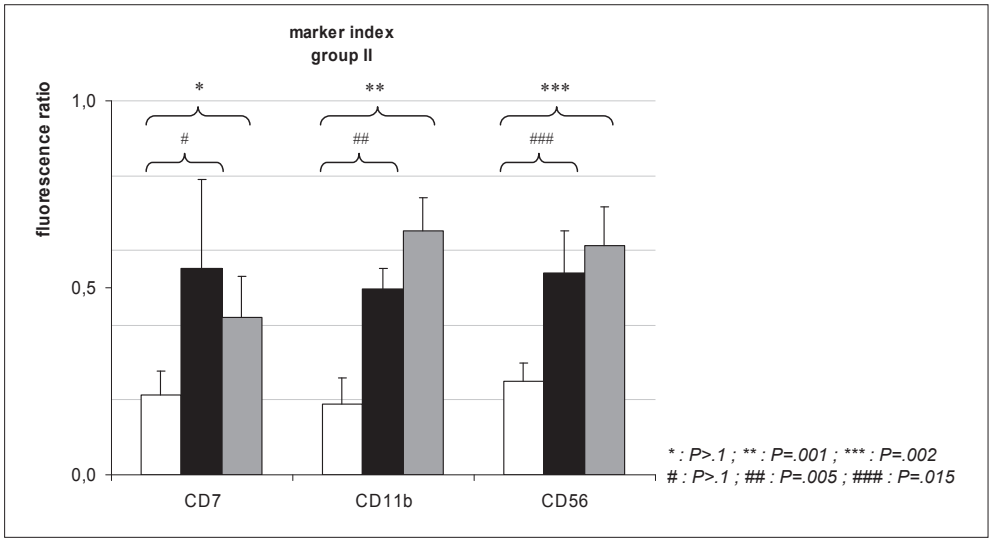


Figure 5. *Lineage marker expression in CD34/CD45 and FSC/SSC defined subpopulations in patient group II.*

White bars: $CD34^{low}/CD45^{low}/FSC^{low}/SSC^{low}$; black bars: $CD34^{low}/CD45^{low}/FSC^{high}/SSC^{high}$; grey bars: $CD34^{high}/CD45^{high}/FSC^{high}/SSC^{high}$.

Differences between the latter two groups ($CD34^{low}/CD45^{low}/FSC^{high}/SSC^{high}$ and $CD34^{high}/CD45^{high}/FSC^{high}/SSC^{high}$) are all NS.

Group III.

Patterns of FSC/SSC and CD34/CD45 expression.

In this group of five patients (patient numbers 20-24, see Table 1) the $CD34^{low}/CD45^{low}$ population was poorly defined and much smaller than the $CD34^{high}/CD45^{high}$ population (see Table 2). Now, the majority (50-100%) of $CD34^{low}/CD45^{low}$ cells were FSC^{high}/SSC^{high}. A representative example of this group is shown in Figure 4B.

Lineage marker expression

Similar to groups I and II, lineage marker expression was lower in the $CD34^{low}/CD45^{low}$ cells than in the $CD34^{high}/CD45^{high}$ cells, but differences were not statistically significant (see Figure 2C). Also similar to group II, within the $CD34^{low}/CD45^{low}$ population, FSC^{high}/SSC^{high} cells had higher expression of CD7, CD11b and CD56 than the few available FSC^{low}/SSC^{low} cells (not shown), although these differences were non-significant. Expression of CD2, CD5, CD19, CD22 or CLL-1 was lacking in all samples (data not shown).

FISH analysis

Sorting of $CD34^{high}/CD45^{high}$ cells of three patient samples revealed 98% (range 95-100%) *BCR-ABL* positivity, but now $CD34^{low}/CD45^{low}$ cells in two patients were for the vast majority *BCR-ABL* positive: 81% and 96%, respectively (see Table 3). In agreement with the results for group II, in these cases the $CD34^{low}/CD45^{low}$ contained in majority FSC^{high}/SSC^{high}: 66% and 86%, respectively. In one patient (no. 20), $CD34^{low}/CD45^{low}$ cells were subsequently sorted on low FSC/SSC: although sorting these very few cells is difficult, still the resulting cells now were for the largest part (71%) *BCR-ABL* negative, again demonstrating that sorting on CD34/CD45 and FSC/SSC more reliably identifies CML and normal stem cells than sorting on CD34/CD45 only. No long term culture assays were performed in this group.

Thus, in agreement with the results of group II, *BCR-ABL* positive stem cells are present, not only in the $CD34^{high}/CD45^{high}$ compartment, but also in the $CD34^{low}/CD45^{low}$ compartment where they make up the majority of this compartent. These cells, which may be lineage marker positive, are however all found in the FSC^{high}/SSC^{high} compartment, together with all $CD34^{high}/CD45^{high}$ cells. In contrast, normal stem cells segregate into $CD34^{low}/CD45^{low}/FSC^{low}/SSC^{low}$ compartment.

Correlations with clinical risk scores.

The very large differences in CML stem cell frequencies as well as the ratio with normal stem cells, might suggest underlying biological differences between patients in the total stem cell compartment possibly related to the stage of disease. We therefore made an inventory of the Sokal and Euro risk scores (Figure 6). These differed significantly in the three groups, especially between group I and III. Group I patients had lowest risk scores, while group III patients had highest. Because follow-up was relatively short for most patients we could not assess whether patients in the different groups had significant differences in cytogenetic or molecular response rates.

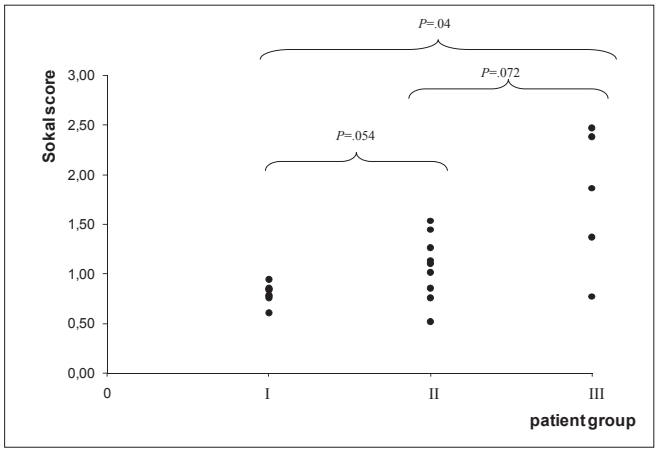


Figure 6A. *Sokal scores in groups I, II and III.*

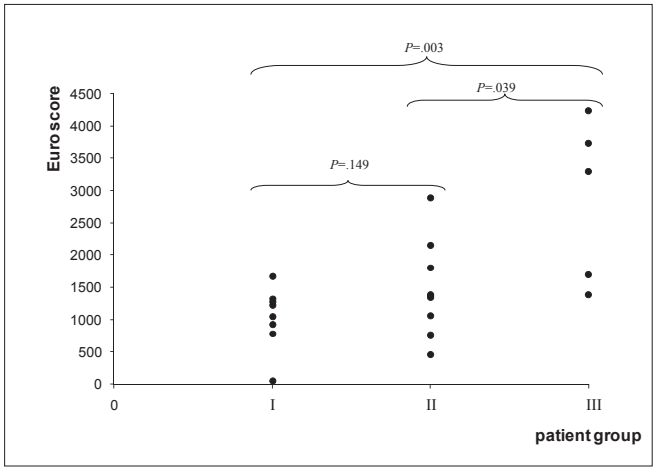


Figure 6B. *Euro scores in groups I, II and III.*

Discussion

Previously, Philadelphia positive and negative long term culture initiating cells in primary CML bone marrow or blood could only be identified indirectly by performing FISH analysis or RT-PCR on colonies that had grown out of remaining viable cells after at least five weeks of culture.[20,21] In this report, we demonstrate for the first time that CML stem cells from individual patients can be distinguished from normal stem cells by flowcytometry in a direct way. In 24 patients, we tested whether flowcytometric parameters would enable to discriminate CML from normal hematopoietic stem cells that both populate the stem cell compartment in CML bone marrow. In 9/24 patients, a $CD34^{low}/45^{low}$ population was also FSC^{low}/SSC^{low} and highly enriched for *BCR-ABL* negative cells, while $CD34^{high}/CD45^{high}$ cells were also FSC^{high}/SSC^{high} and highly enriched for BCR-ABL positive cells. In the other two patient groups there was increasing contamination of the $CD34^{low}/CD45^{low}$ compartment with BCR-ABL positive cells. These cells were nonetheless, and similar to $CD34^{high}/CD45^{high}$ cells, high in FSC and SSC. Accordingly, the BCR-ABL negative normal compartment, i.e. $CD34^{low}/CD45^{low}/FSC^{low}/SSC^{low}$, dramatically decreased in size. In all patients, the population that was both $CD34^{high}/CD45^{high}$ and FSC^{high}/SSC^{high} was always BCR-ABL positive, showing that the CML part of the stem cell compartment in CML bone marrow can always be identified.

The data presented here is relevant in view of several issues. *Firstly*, the ability to discriminate malignant from benign stem cells within a single patient will make molecular and functional characterization of the CML stem cell compartment, e.g. by gene expression array studies, much more informative. This is especially important when normal stem cells make up the majority of the total stem cell compartment as was the case in patient group I. Background "noise" due to mixing of leukemic and non-leukemic stem cells as well as to interindividual differences in non-relevant gene expression can now be reduced by accurate FACS sorting of separate populations. This may improve development of treatments with an optimal therapeutic window as now the absence of putative targets in or on the simultaneously present normal stem cells can be taken into account.

Secondly, quantitation of the malignant and benign stem cells in CML may have clinical value in addition to the already used prognostic indices like the Sokal- and Euro-scores. In clinical trials, lower Sokal or Euro-risk scores were associated with higher response rates to both conventional treatment and to tyrosine kinase inhibition.[5,19] Remarkably, we found significantly lower Sokal and Euro risk scores in patients with a large normal stem cell population as defined by the CD34/CD45 and FSC/SSC characteristics (low for both) as compared to those with a large CML stem cell population (FSC^{high}/SSC^{high}). These results suggest that the clinical risk scores are associated with the residual presence of normal stem cells. Unfortunately, we were unable to adequately analyze our cohort of patients with respect to response, since follow-up was rather short.

Residual leukemic stem cell enumeration may also serve as a relevant surrogate end point in clinical studies with new (stem cell targeting) drugs. Furthermore, clinical CML management might in future be guided by detection of normal and residual leukemic stem cells. As approximately 50% of imatinib treated patients with long-lasting complete molecular responses maintained their response after stopping imatinib, it would be highly relevant to see whether persistent remission could be predicted by flowcytometric analysis of the primitive stem cell compartment.[22] In view of this question, a Dutch HOVON study has been initiated in patients with long lasting complete molecular responses. After interruption of imatinib, regular evaluation of the $CD34^{+}CD38^{-}$ stem cell compartment using our flowcytometric strategy, together with quantitative PCR on *BCR-ABL*, will be performed to guide re-initiation of imatinib in these patients.

The *third* relevant issue is that in most studies, CML stem cells are defined by their lineage negativity.[10,14,23] We however showed that long term culture initiating leukemic cells may show variable expression of the lineage markers CD7, CD11b and CD56. Lineage marker negative stem cells therefore only constitute a subpopulation of the leukemic stem cells that have long term clonogenic potential. Possibly, these cells differ in more characteristics (in gene or protein expression, biological behavior) from their marker positive counterparts. Using only marker negative cells for in vitro or in vivo experiments may thereby bias results. Lineage marker positivity was a property almost exclusively characterizing FSC^{high}/SSC^{high} BCR-ABL positive cells, thereby resembling the AML stem cell compartment.[16]

In conclusion, flowcytometric discrimination of leukemic and normal stem cells is an exclusive tool for defining new therapeutic targets and applied diagnostics in CML. We are now exploring this technique for the analysis of differentially expressed genes in malignant versus benign stem cells and are planning in-vivo experiments with leukemic stem cells that have been identified by flowcytometry.

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7

RAPID COMPLETE CYTOGENETIC REMISSION AFTER UPFRONT DASATINIB MONOTHERAPY IN A PATIENT WITH A NUP214-ABL1-POSITIVE T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA.

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Abstract

The cryptic NUP214-ABL1 rearrangement in T-cell acute lymphoblastic leukemia (T-ALL) was only recently identified as a new oncogene involved in T-ALL. Ensuing constitutively activated tyrosine kinase activity has provided a rationale for treatment with specific kinase inhibitor therapy, whereby the more stronger inhibitors may be preferred as a result of the extensive *ABL1* amplification. Here, we present a patient with a NUP214-ABL1-positive T-ALL, who was initially treated with dasatinib monotherapy prior to combination chemotherapy and continued dasatinib. The patient achieved a complete hematologic and cytogenetic remission after three weeks of dasatinib monotherapy, followed by a complete immunological and molecular remission upon induction chemotherapy in combination with dasatinib. Following the harvest of a NUP214-ABL1-negative hematopoietic stem cell graft, the patient was treated by autologous transplantation followed by dasatinib maintenance. His molecular remission has persisted until the most recent follow-up, at 9 months after diagnosis. This observation clearly shows the clinical efficacy of kinase inhibitor therapy in NUP214-ABL1-positive T-cell ALL and provides the rationale for further study with dasatinib in combination with chemotherapy in this category of ALL.

Introduction

Enhanced tyrosine kinase activity due to constitutively activated ABL1 plays an important role in several leukemias. The *BCR-ABL1* fusion gene is the cytogenetic hallmark of chronic myeloid leukemia (CML) and is also frequently observed in B-cell acute lymphoblastic leukemia (ALL), but is exceptionally rare in T-ALL.[1-3] However, amplification of *ABL1* may be observed in up to 6% of T-ALL patients, as was initially described by Barber et al.[4] Amplification of *ABL1* appeared to be due to a cryptic episomal fusion of NUP214 to ABL1, which was first described by Graux et al.[5] Apart from fluorescence in situ hybridization (FISH) to visualize the *ABL1* on the episomes, real-time quantitative polymerase chain reaction (RQ-PCR) may be used for diagnosis and monitoring of response and minimal residual disease (MRD).[6,7]

Targeting of the pathogenetically relevant BCR-ABL1 tyrosine kinase with the kinase inhibitor imatinib has been a major breakthrough in the management of patients with CML and Philadelphia-positive ALL. *In vitro*, the constitutively activated tyrosine kinase NUP214-ABL1 appeared sensitive to the tyrosine kinase inhibitor imatinib in T-ALL cell lines carrying the *NUP214-ABL1* transcript.[5] This finding provided the rationale to introduce kinase inhibitor therapy in the treatment of patients with this specific fusion gene. So far, only one patient with a relapsed NUP214-ABL1-positive T-ALL failing imatinib monotherapy, was described.[8] Given the possibility of imatinib resistance because of extensive amplification of *ABL1* and the more potent inhibition exerted by dasatinib (formerly BMS-354825), we hypothesized that dasatinib may be preferred for treatment of NUP214-ABL1-positive ALL. Here, we describe the first patient with a NUP214-ABL1-positive T-ALL, who favorably responded to dasatinib monotherapy. This report provides the rationale to introduce the more potent kinase inhibitors in the treatment of NUP214-ABL1-positive T-cell ALL.

Materials and methods

Cytogenetic and FISH analysis

Bone marrow and blood were cultured and harvested according to standard cytogenetic protocols, both at diagnosis and follow-up. At diagnosis 25 metaphase cells were analysed using both QFQ- and RBA-banding. The resulting karyotype was described according to the International System for Human Cytogenetic Nomenclature (ISCN 2005).[9] Fluorescence in situ hybridization was performed using standard protocols.[10] The presence of MLL translocations was determined using the LSI MLL Dual Color, Break Apart Rearrangement Probe (Vysis, Abbott Molecular, Abbott Park, Illinois, USA). The presence of BCR-ABL1

translocation or amplification of ABL1 was investigated using the LSI BCR/ABL ES Dual Color Translocation Probe (Vysis). At least 200 interphase nuclei per sample were analyzed using an epi-fluorescence microscope (Zeiss, Axio-Imager Z1, Sliedrecht, The Netherlands) and Isis Software (Metasystems, Altlussheim, Germany)

Real-time quantitative NUP214-ABL1 PCR (RQ-PCR)

Total RNA was extracted form peripheral blood and bone marrow using RNABee (Campro Scientific, Veenendaal, The Netherlands). cDNA was synthesized from 1 μg of RNA using random hexamer priming, essentially as described.[11] cDNA prepared from 50 ng or 25 ng of RNA was used for the PCR amplifications to determine *NUP214-ABL1* and porphobilinogen deaminase (*PBGD,* reference gene) transcript levels, respectively. *NUP214-ABL1* levels were determined using forward primer NUP214 5'-CCTCTGGGTTCAGCTTTT-3', reverse primer T.BA REV 3'-TCAGACCCTGAGGCTCAAAGTC-5' and probe BCR-ABL1 6-FAM 5'-AAGCCCTTCAGCGGCCAGTAGCA-3'TAMRA and *PBGD* levels using forward primers PBGDrev 5'-GGGTACCCACGCGAATCAC-3', reverse primer PBGDforw 5'- GGCAATGCGGCTGCAA -3' and probe PBGD 6 - F A M 5'- CATCTTTGGGCTGTTTTCTTCCGCC - 3'TAMRA. RQ-PCR amplifications were performed using the ABI 7500 real time PCR system (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) using 25 μL RQ-PCR mixes containing 200 μM [*NUP214-ABL1*] or 125 μM [*PBGD*] deoxyribonucleoside triphosphates (dNTPs; Amersham Pharmacia Biotech, Roosendaal, The Netherlands); 7.5 pmol [*NUP214-ABL1*] or 3.75 pmol [*PBGD*] forward and reversed primer (Invitrogen, Breda, The Netherlands); 2 mM [*NUP214-ABL1*] or 5 mM [*PBGD*] $MgCl_2$; 1.36 pmol [*NUP214-ABL1*] or 1.25 pmol [*PBGD*] probe, labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3'end with the quencher dye molecule TAMRA (6-carboxy-tetramethylrhodamine) (Eurogentec, Maastricht, The Netherlands), 5 μL 10 x buffer A and 1.25 [*NUP214-ABL1*] or 0.63 U [*PBGD*] AmpliTaq Gold (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). The thermal cycling conditions for *NUP214-ABL1* and *PBGD* included 10 minutes at 95°C followed by 45 cycles of denaturation for 20 seconds at 95°C, annealing at 58°C for 20 seconds and extension at 60°C for 30 seconds. A dilution series of RNA of the diagnostic bone marrow sample in RNA of HL60 was used to determine the expression levels of *NUP214-ABL1* relative to the diagnostic bone marrow sample. The *NUP214-ABL1* quantities were normalized using the endogenous reference gene *PBGD* (Normalized quantity = Quantity dilution series x $2^{(\text{Ct PBGD sample} - \text{Ct PBGD sample bone marrow at diagnosis})}$). The reproducible sensitivity of the assay was 10^{-4} (bone marrow at diagnosis/ HL60). All samples were analyzed in duplicate.

Case report

A 21-year old man with a 3-day history of progressive abdominal pain was referred to the emergency ward. There was no history of abdominal trauma, no recent infections were reported and no fever had been noticed. He also denied nausea, vomiting, or alteration in bowel or bladder habits. Physical examination showed an acute ill patient with a temperature of 39 ˚C. He was hypotensive and abdominal examination revealed 'defense musculaire', suggesting peritoneal inflammation (acute abdomen). Laboratory findings at admission included a hemoglobin level of 6.4 g/dL, a white blood cell count (WBC) of 41 x 10^9/l with 87% of lymphoblasts in the differential and a platelet count of 20 x 10^9/l. Following transfusion of erythrocytes, platelets and fluids, the patient was stabilized and offered for surgery. At laparotomy, rupture of an enlarged spleen was found, which necessitated a splenectomy.

Meanwhile, morphology and immunophenotyping of blood and bone marrow showed malignant blasts positive for CD45, TDT, cyCD3, CD5, CD2, CD1a, CD4, CD8 and CD10, without expression of other B-lymphoid markers or myeloid markers. These findings established a diagnosis of a common T-cell ALL. Histology of the spleen showed extensive infiltration of T-lymphoblasts and further examination by a computed tomography (CT) scan showed an enlarged mediastinum and mediastinal and abdominal lymphadenopathy. Cytogenetics revealed no abnormalities, but extrachromosomal amplification of *ABL1* was demonstrated by FISH (Figure 1), suggesting a *NUP214-ABL1* fusion, which was subsequently confirmed by RQ-PCR. Given the FISH findings, and a protracted abdominal recovery after surgery, immediate start of intensive combination chemotherapy was postponed, and the patient was started with dasatinib 70 mg twice daily (Table 1), following informed consent. After 3 weeks of treatment with dasatinib monotherapy, the patient achieved a complete hematologic and complete cytogenetic remission. Assessment of MRD by flowcytometry and by quantitative PCR showed 0.4 % residual T-lymphoblasts and a reduction of 2-log of *NUP214-ABL1* copies as compared to baseline level, respectively. At that time, the patient had recovered from surgery and induction chemotherapy was started (vincristine, prednisone and daunorubicine according to the HOVON-37 protocol, but without asparaginase), in combination with dasatinib. Dasatinib was very well tolerated throughout the treatment course and no side effects were noted. Following consolidation with cytarabine and mitoxantrone, consolidation with high-dose methotrexate and asparaginase was given, whereas dasatinib was temporarily interrupted during the phase of asparaginase. Side effects of chemotherapy included two episodes of neutropenic fever, and transient common toxicity criteria grade 1-2 mucositis. A complete immunological and molecular remission ensued after the first consolidation course of chemotherapy (Figure 2). At the time of initial neutrophil recovery following the first consolidation, granulocyte colony-stimulating factor (G-CSF) was given for mobilization of peripheral blood stem cells (PBSC) and the harvest of a PBSC-graft. A sufficient number of CD34-positive progenitor cells were harvested and the molecular evaluation of the graft did not reveal MRD by RQ-PCR. Lacking a matched

sibling donor, the patient qualified for autologous stem cell transplantation (SCT) according to the HOVON-37 protocol, which was performed at 6 months from diagnosis following myeloablative conditioning with cyclophosphamide and total body irradiation (12 Gy in 2 fractions). The post-transplant course was uneventful and following full hematologic recovery, the patient was restarted on dasatinib, which will be continued as maintenance therapy for 2 years. His latest bone marrow examination at 3 months after transplantation showed persistent molecular remission.

Table 1. *Treatment of the patient and response on treatment*

Days after start dasatinib	Treatment	Blood	Bone marrow			
		WBC (10^9/l)	Morphology blasts (%)	Immunology MRD (%)	FISH (%)	Molecular (%)
-4	Baseline	21	90	85	84	-
0	Dasatinib 2 x 70 mg	27	-	-	-	100
20	Evaluatio	6.0	2	0.4	0	1
24	Chemotherapy 1e course with dasatinib	7.3	-	-	-	-
52	Evaluation	7.4	2	<0.05	0	<0.01
59	Chemotherapy 2nd course with dasatinib	5.6	-	-	-	-
96	Evaluation	6.8	1.2	<0.05	0	negative
104	Chemotherapy 3rd course with dasatinib	5.7	-	-	-	-
138	Evaluation	3.9	0.2	<0.05	0	negative
161	Autologous transplantation	1.6	-	-	-	-
257	Evaluation	6.1	1	<0.05	0	negative

Abbreviations: WBC, white blood cell count; MRD, minimal residual disease; FISH, fluorescence in situ hybridization

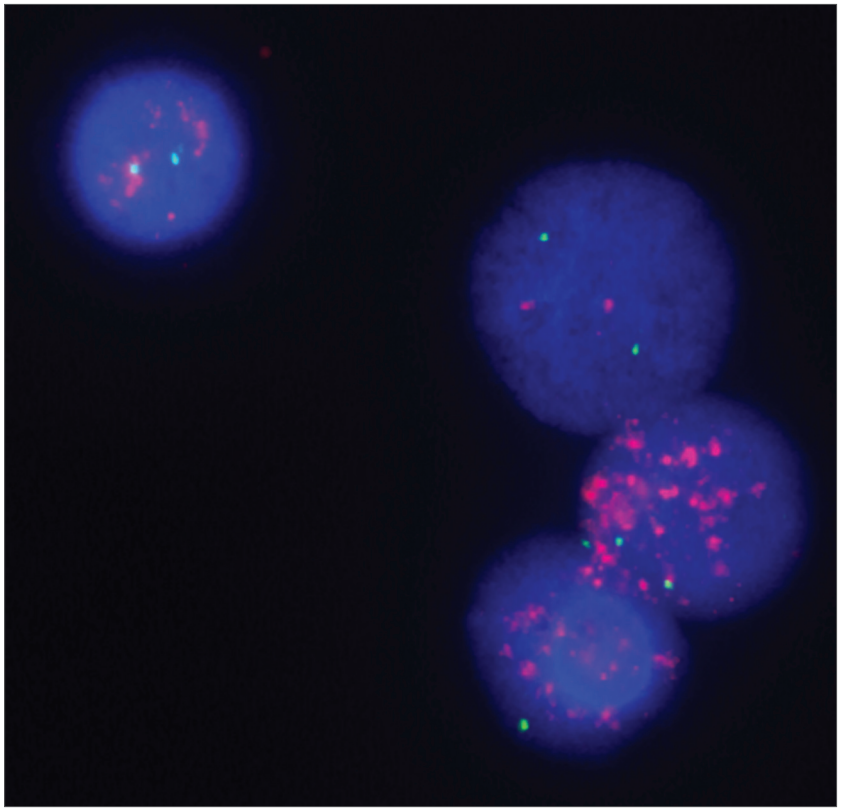

Figure 1. *Fluorescence in situ hybridization using probes for BCR (green signal), and ABL1 (red signal) showing the extrachromosomal amplification of ABL1.*

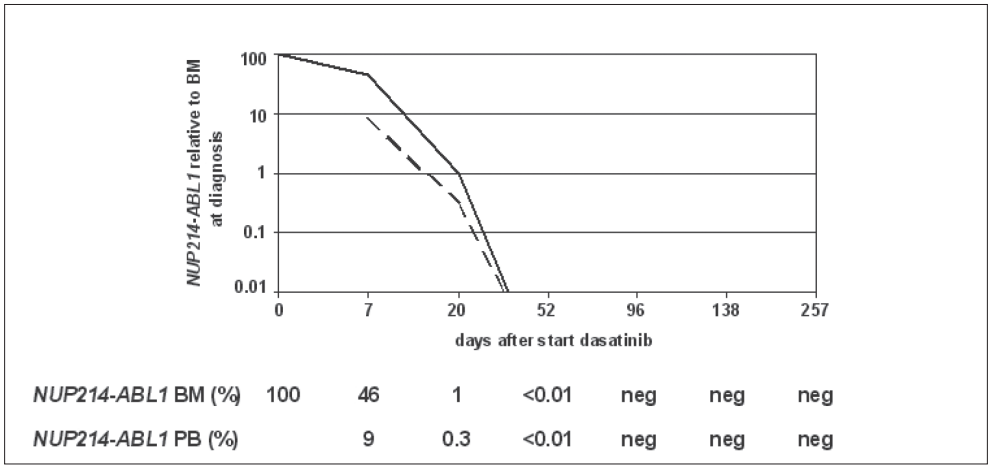

Figure 2. *Molecular response in blood and bone marrow from the introduction of dasatinib in the NUP214-ABL1-positive patient.*

Discussion

Constitutively activated tyrosine kinases may be involved in the pathogenesis of several myeloid and lymphoid leukemias. Examples of hematologic malignancies, in which constitutively activated tyrosine kinases play a major pathogenetic role, BCR-ABL1 in CML and ALL, PDFGR-α in the hypereosinophilic syndromes, FLT3 in acute myeloid leukemia, and NUP214-ABL1 in ALL as was also present in this case.[13,14] Although BCR-ABL1 and NUP214-ABL1 share a common tyrosine kinase domain, there are also many differences including a different activation state and conformation of the two ABL1 fusion kinases, substrate preference and inhibitory sensitiviy as was recently reported.[15] Analysis of the transforming properties and structure-function studies have enabled the development of small molecules that specifically inhibit activated kinases. Imatinib mesylate was the first of these drugs that has entered clinical medicine, and has revolutionized the treatment of CML.[16,17] Other inhibitors of BCR-ABL1 include nilotinib and dasatinib.[18-20] Nilotinib (formerly AMN107) exerts a higher and more specific binding affinity for the ABL1 kinase, appeared active against many imatinib-resistant ABL1 mutants, but binds ABL1 kinase in its inactive conformation only. Dasatinib on the other hand binds ABL1 both in its active and inactive conformation, resulting in a 10-fold higher potency than nilotinib and is active against most imatinib-resistant ABL1 kinase domain mutants. In addition, dasatinib also inhibits the kinase activity of many other kinases, including the SRC family. LCK, a member of the SRC family of tyrosine kinases, is highly expressed in T-cells and plays an important role in the initiation of the T-cell receptor-signaling pathway.[21,22] De Keersmaecker et al. showed that LCK was critical for NUP214-ABL1 signaling, and they suggested that LCK was an interesting alternative target in NUP214-ABL1-positive patients, when they were searching for other therapeutic targets in these patients.[23] Recently, Quintas-Gardama et al. examined and compared the activity of imatinib, nilotinib, and dasatinib in several human NUP214-ABL1-positive T-cell lines as well as patient derived lymphoblasts.[24] Altough all 3 inhibitors showed inhibition of cellular proliferation and induction of apoptosis, especially dasatinib appeared associated with strong apoptotic effects, and inhibition of phosphorylation. Moreover, a significant prolongation of survival was observed in a murine xenograft leukemia model following treatment with dasatinib as compared to placebo. Therefore, based on these findings as well as on the demonstrated inhibitory activity against the SRC-kinases, dasatinib may be preferred as an inhibitor in NUP214-ABL1 T-ALL patients. These considerations are now strongly underpinned by the striking clinical activity as observed in our patient. Applied as monotherapy, dasatinib rapidly induced a complete hematologic and complete cytogenetic response, followed by a complete molecular response upon continuation of dasatinib in combination with standard chemotherapy. The experience with tyrosine kinase inhibitors in patients with a NUP214-ABL1-positive T-ALL is very limited. So far, only one patient with a NUP214-ABL1-positive T-ALL was reported, who received imatinib monotherapy for his first relapse, but failed to respond.[8]

Our results suggest that every effort has to be taken to establish an early diagnosis of the episomal NUP214-ABL1 amplification in patients with T-ALL, which is to be followed then by incorporation of dasatinib in the chemotherapy schedules. Preferably, a combination of dasatinib and chemotherapy should be given. Although the presence of the NUP214-ABL1 fusion was not associated with a very poor outcome in the German series of patients, overall outcome was still unsatisfactory and estimated approximately 50% at 5 years.[6] Addition of dasatinib may be expected to improve the outcome of these patients considerably, but an improvement of approximately 20% might already be considered as a major step forward. It implies that a reduction of the intensity of the current chemotherapy should be considered as too early and mature results of multiple patients with this particular subset of T-ALL is needed. What about the application of SCT? Allogeneic stem cell transplantation (allo-SCT) from a sibling donor was recently shown to improve outcome in adult ALL, including poor-risk and standard-risk patients.[25] Result reported by Burmeister et al. suggest that NUP214-ABL1 ALL may be considered as standard-risk ALL and may, therefore, qualify for allo-SCT from a sibling donor. It is, however, questionable whether unrelated allo-SCT should be explored for NUP214-ABL1 ALL patients in first complete remission lacking a matched sibling donor. Alternatively, an allograft with a matched unrelated donor could be offered in second remission for relapsing patients. Moreover, the *NUP214-ABL1* fusion transcript can be followed quantitatively, allowing identification of early molecular relapses, which could then be followed by allo-SCT. We applied a relatively short and intensive course of chemotherapy consisting of 3 cycles followed by an autograft after myeloablative conditioning. The use of autologous SCT in ALL, as applied in our patient, has been debated repeatedly. The MRC-ECOG study recently showed no benefit of autologous SCT in standard-risk ALL patients as compared to continued chemotherapy.[25] However, the introduction of myeloablative irradiation offers the advantage of introducing radiotherapy in patients with T-ALL, who often present with extensive extramedullary leukemia, as was observed in this case. Similar to patients with BCR-ABL1-positive B-ALL, we choose to continue dasatinib maintenance guided by regular monitoring of residual disease by PCR every 3 months, which may enable a diagnosis of an imminent relapse.

In conclusion, FISH with probes for *ABL1* should be included in the work-up of T-ALL patients, to rapidly demonstrate the presence of the *NUP214-ABL1* episomal amplification. Based on extensive *in vitro* findings[23,24] as well as the present case report, dasatinib may then be the preferred inhibitor to be combined with standard chemotherapy schedules. The inclusion of these patients into an international protocol standardizing treatment allows to address important clinical and biological questions in this particular and rare subset of T-ALL.

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8
GENERAL DISCUSSION

Introduction

Since the turn of the century, prospects for patients with chronic myeloid leukemia (CML) have dramatically changed. While interferon alfa (IFN-) or allogeneic hematopoietic stem cell transplantation (allo-SCT) were the cornerstones of therapy in the years 1980-2000, currently all newly diagnosed CML patients receive tyrosine kinase inhibitor therapy as a first-line of treatment. Although improved survival by intention-to-treat could not be demonstrated in the pivotal prospective randomized study comparing imatinib versus combination therapy consisting of IFN-α and cytarabine due to extensive cross-over, it is now generally accepted that imatinib treatment is associated with a dramatic and sustained survival benefit for first chronic phase CML patients. The HOVON cooperative group performed 2 successive clinical trials since 1998, which form the basis of this thesis. The first study (HOVON-38) addressed the question whether intensified cytarabine followed by IFN-α would be superior to low-dose cytarabine combined with IFN-α. The second study (HOVON-51) addressed the question whether combination therapy of imatinib with cytarabine would be feasible and improve outcome in CML patients in first chronic phase. While low-dose cytarabine was considered an essential part of therapy before the introduction of imatinib, it is currently not recommended as part of first-line therapy. Therefore, first, the role of cytarabine will be critically addressed in view of the 2 HOVON studies as well as current literature.

The introduction of imatinib since 2001 was accompanied by a rapid evolution in molecular diagnostic techniques, enabling us to more closely monitor therapy and develop parameters for prediction of outcome. The current place of molecular diagnostics will be discussed, especially from a clinical perspective.

Lastly, this general discussion will highlight the development of the second generation of tyrosine kinase inhibitors, including a critical assessment of their place in our current therapeutic armentarium.

1. Combination therapy of imatinib and cytarabine

Before the introduction of imatinib, patients with CML in first chronic phase were preferably treated with a combination of IFN-α and low-dose cytarabine. The addition of cytarabine to IFN-α had been shown to improve the major cytogenetic response (MCR) from 24% to 41%, as compared to IFN-α alone in a randomized phase III study.[1] Furthermore, a survival benefit of approximately 6% was observed with the combination of IFN-α and cytarabine. As a result, the combination of IFN-α and cytarabine became standard therapy for patients with chronic phase CML. In studies exploring the efficacy of intensive chemotherapy, a MCR was observed in 30-60% of patients, but these responses were transient without maintenance therapy.[2,3] Given the dose-response effect of cytarabine in other myeloid leukemias, e.g., acute myelogenous leukemia (AML), a prospective phase III study (HOVON-38) was performed to evaluate whether intensified cytarabine followed by IFN-α would induce a higher response rate and longer event-free interval as compared to low-dose cytarabine combined with IFN-α in early chronic phase CML (Chapter 2). Early cytogenetic response rates appeared higher with intensified cytarabine, but most responses were rapidly lost and event-free survival (EFS) proved similar between the 2 treatment arms.[4] These results suggested that the addition of intensified cytarabine may have resulted in the eradication of more mature, differentiated malignant myelopoiesis, but clonogenic precursors were suggested to remain unaffected. Of note, survival at 5 years estimated 77% and 56% in the low-dose and high-dose arm, respectively, whereas EFS at 3 years estimated 37% and 23% in the HOVON-38 treatment arms. These results underscore the benefit obtained with imatinib in recent years, as 5-year survival was > 90% in the HOVON-51 study. That benefit may even be underestimated, because 44 patients (73%) entered in the HOVON-38 study received imatinib as second- or third-line treatment and may thereby have gained from tyrosine kinase inhibitor therapy. The latter suggestion is supported by the large difference in event-free and overall survival in that study, which differs approximately 40% (Figures 1 and 2, Chapter 2).

Imatinib at a dose of 400 mg daily is currently considered standard treatment in patients in early chronic phase CML. In the International Randomized Study of Interferon and STI571 (IRIS) a cumulative incidence of complete hematologic response (CHR) and complete cytogenetic response (CCR) was observed in 98% and 87%, respectively, at 5 years.[5] Complete molecular response (CMR) occurred infrequently.[6] Furthermore, with a median follow-up of 60 months only 6% of the patients had progressed to advanced phase CML and the estimated overall survival (OS) was 89% at 60 months. No meaningful comparison with the control group was possible, because only 3% of patients still continued initial treatment with IFN-α and low-dose cytarabine. Of note, in reports of this study approximately

15% - 20% of patients in the imatinib-group were censored for various reasons including insufficient response and intolerance of treatment. End points including response rates, progression to advanced phase, and EFS were selectively evaluated for patients continuing imatinib. The results of the IRIS study may therefore differ from what can be observed when an intention-to-treat analysis is performed. The Hammersmith Hospital reported their experience in 204 patients with newly diagnosed CML in chronic phase using an intention-to-treat analysis.[7] The 5-year cumulative incidences of a CCR and major molecular response (MMR) were 83% and 50%, respectively. A CMR occurred again infrequently and was observed in 8% of patients only. Moreover, EFS as defined by a stable cytogenetic response while still receiving imatinib was 63% at 5 years. The EFS appeared relatively disappointing, because a considerable number of patients stopped imatinib treatment due to progression, unsatisfactory response, and/or intolerance of treatment. The latter results have clearly highlighted that further improvement should be pursued.

In order to improve the molecular response rate and to prevent resistance the HOVON cooperative group set out to explore the feasibility and efficacy of combination therapy of 4 different dosages of imatinib (200 mg, 400 mg, 600 mg or 800 mg) and 2 different dosages of cytarabine (200 mg/m^2 or 1000 mg/m^2) intravenously. The feasibility results of that combination are presented and discussed in Chapter 3. While a higher dose of cytarabine was associated with more infectious complications, the combination of a standard-dose of intravenous cytarabine with different dosages of imatinib appeared to be tolerated very well.[8] With a median follow-up of 55 months, efficacy was analysed, which is described in Chapter 4. The 5-year cumulative incidences of a CCR and CMR were 89% and 53%, respectively. Overall survival at 5 years was 96% and EFS, similarly defined as in the Hammersmith-study, was 69%. Given a rather comparable risk profile of patients entered, these results may suggest that combination therapy may result in better outcome and at least result in a better CMR at 5 years.

By inducing an early molecular response, combination therapy may prevent primary hematologic or cytogenetic resistance, and it may also prevent secondary resistance or progression in patients relapsing from an earlier established response. Given the high rates of hematologic and cytogenetic responses observed in the IRIS-study, the Hammersmith-study, and our study and the low rates of progression in either study, it is difficult to draw firm conclusions from small differences. Given the design of the HOVON-51 study as a phase II/feasibility study, no definite conclusions with respect to the role of cytarabine can be drawn. However, a higher dose of cytarabine appeared associated with an approximately 10% higher CMR rate as compared to the standard-dose of cytarabine in our study. Furthermore, none of the patients receiving the higher dose of cytarabine has shown progressive disease until the latest follow-up. These results do suggest at least an additive effect of cytarabine. However, only a direct comparison in a prospective randomized trial may answer the question to what extent cytarabine may improve the molecular response rate and prevent disease progression.

Therefore, the HOVON cooperative group recently started a prospective randomized study comparing 800 mg imatinib versus the combination of 800 mg imatinib with cytarabine at a dose of 200 mg/m^2 intravenously (HOVON-78). In conclusion, the introduction of imatinib has been a major advantage in the treatment of patients with CML. However, approximately one third of patients will need other treatment because of intolerance or resistance. Combination therapy consisting of imatinib and cytarabine has yielded promising results as regards molecular response and prevention of resistance. However, no definitive conclusions can be drawn, necessitating a randomized trial (HOVON-78), which is currently under way and may definitely settle the contributory role of cytarabine.

2. Molecular monitoring

The introduction of molecular techniques for identifying and measuring *BCR-ABL* transcripts has enabled a more exact assessment of response to therapy in CML. Initial polymerase chain reaction (PCR) techniques only allowed to identify the presence or absence of *BCR-ABL* transcripts by a single-step amplification.[9] Second generation PCR techniques applied a so-called 2-step or "nested" amplification with internal primers to increase the sensitivity.[10] The methodology used for measuring *BCR-ABL* transcripts has improved over the years. Important contributions were made by Cross et al. and Hochhaus et al., who introduced the quantitative assessment of transcript numbers.[11,12] Many molecular laboratories developed their own method for molecular monitoring and considerable diversity in the way in which *BCR-ABL* copies were quantified arose. Thereby, it became difficult to compare molecular responses in different studies. A first attempt to harmonize quantitative PCR (Q-PCR) was made by Hughes and other investigators of the IRIS study with the concept of expressing results as log reduction compared to a standardized baseline of untreated patients. In a further attempt to harmonize and compare molecular results, an international scale was developed. A conversion factor for each laboratory was derived by comparing samples, and this factor enabled converting individual molecular results to the international scale to be expressed as percentage reduction as compared to a standardized control, referred to as 100%.[13,14] *BCR-ABL* values below 1% on the international scale appeared very well correlated with CCR as was demonstrated by Ross et al.[15] A *BCR-ABL* value of 0.1% on the international scale corresponds to a MMR, as was previously defined by Hughes et al.[6,13,14] Achieving a MMR correlated with superior outcome in the IRIS study as not a single patient that attained a MMR at 18 months had progressed at 5 years,[5,6] Subsequently, achieving a MMR became an important goal. What is the advantage of achieving a MMR instead of a CCR? Our results, as well as those by several others, clearly suggest that patients with a more profound response, benefit in terms of durability of CCR, less disease progression and prolonged PFS.[5,6,16-18] Of note, progression was completely absent in the HOVON-51

study, who had developed a MMR at 1 year (Figure 6, Chapter 5). The HOVON-51 study, however, did not show a survival difference between patients achieving a CCR versus those obtaining a MMR. While a MMR is currently expressed as 0.1% on the international scale, a CMR is defined by no residual *BCR-ABL* transcripts by RQ-PCR with a level of sensitivity of the assay of at least 0.01%. A negative RQ-PCR has to be accompanied by a negative nested PCR, the latter which may be slightly more sensitive as RQ-PCR, when more stringent criteria to define a CMR are used. Should we pursue a CMR nowadays? Recent preliminary reports seem to suggest that some patients with a CMR may possibly be cured, as evidenced by absence of molecular relapse following cessation of imatinib maintenance. Rousselot et al. initially described 6 out of 12 patients in CMR who were able to discontinue imatinib without disease recurrence.[19] A comparable observation was done by Hughes et al. with approximately 50-60% of patients being able to discontinue imatinib without disease recurrence.[20] However, longer follow-up may be needed to definitely assess to what extent patients may be cured. However, these studies for the first time suggest that apart from allo-SCT, also "conventional" treatment based on tyrosine kinase inhibition may be associated with cure and the pursuit of a CMR may indeed become a future goal of therapy.

In conclusion, molecular monitoring of *BCR-ABL* has become a sensitive, reproducible technique that allows for close monitoring of the individual patient. Furthermore, molecular monitoring is much more convenient than cytogenetic evaluations. In addition, molecular results in blood and bone marrow do not differ and therefore allow to selectively focus on peripheral blood samples for monitoring individual patients beyond the level of a CCR. It also raises the question how and when to continue conventional cytogenetic analysis. At diagnosis, a cytogenetic evaluation of bone marrow is required to identify any unusual translocations or additional cytogenetic abnormalities. In addition, marrow cytogenetics has to be repeated every 6 months during follow-up until CCR is achieved in order to diagnose possible cytogenetic evolution. In patients with a stable CCR, however, continued cytogenetic evaluation has become debatable. Information that may be missed includes additional cytogenetic abnormalities, that may arise in Philadelphia negative metaphases, which have been reported to occur infrequently and may possibly be related to myelodysplasia.[21,22] On the other hand, if *BCR-ABL* transcripts increase and resistance is suggested, cytogenetic analysis is again required. Furthermore, in these circumstances direct sequencing of the ABL kinase domain is recommended to detect kinase domain mutations.

Kinase domain mutations

At least 70 different mutations involving 57 different amino acids have been reported in the *BCR-ABL* kinase domain. Especially, the T315I mutation and some mutations within the P-loop of *BCR-ABL* are clearly associated with resistance against imatinib.[23] Currently, there is no universal accepted consensus when patients should be screened for mutations.[13,24] In the HOVON-51 study, patients who did not develop a MMR at 1 year were routinely screened for mutations in the *ABL* kinase domain (Chapter 4), which was repeated during follow-up as long as patients failed to achieve a MMR. Patients, who lost their initial response or progressed during follow-up, were also evaluated for mutations. It resulted in a low cumulative incidence of mutations of 10% at 5 years. In total, 14 different mutations were detected in 15 patients. The T315I mutation, found in 2 patients, was strongly associated with clinical resistance to imatinib. Mutations were associated with resistance in 12 out of these 15 patients in the HOVON-51 study, including 3 patients, who also developed clonal evolution. Which patients should routinely be screened for point mutations in the kinase domain? Obviously, patients with primary hematologic or cytogenetic resistance should undergo a thorough evaluation including a mutation screen, as these patients are at high risk of progression and a choice for second-line tyrosine kinase inhibitor therapy should be based on both the type of mutation and whether or not cytogenetic evolution is present.[13,24] While the frequency of mutations is low in chronic phase CML in general,[25] kinase domain mutations are abundant in patients with acquired resistance with reported frequencies of *BCR-ABL* mutations, in between 40% to 90%.[26-28] Differences in observed mutation frequencies may be explained by the definition of resistance, the phase of the disease, but may also depend on the methodology applied. Direct sequencing is associated with a sensitivity of around 20% and may be applied nowadays on a broader scale by specialized laboratories.[13] High-performance liquid chromatography may represent a more sensitive technique for detecting mutations,[29] but apart from the questionable significance of clones with a very low frequency, the method is very laborious.

The clinical significance of finding a particular *BCR-ABL* kinase domain mutation can be complex. Most mutations have been evaluated in vitro for relative sensitivity to imatinib and alternative kinase inhibitors, allowing a therapeutic intervention based on in vitro findings (Table 1). However, resistance may be multifactorial and a number of mutations are associated with only a relative insensitivity to imatinib. For example, in case of associated pharmacological resistance, resistance may sometimes be overcome by first increasing the dose of imatinib. On the other hand, some mutations are functionally irrelevant and therapy with imatinib can be continued.[30] Once patients have acquired a point mutation in the kinase domain, they are probably at increased risk of developing new mutations, and these patients should be carefully monitored.[31] With the approval of dasatinib and nilotinib as second-line tyrosine kinase inhibitors, it has become even more important to define the best clinical approach in individual patients once a mutation has been identified. Choices for second-line tyrosine kinase inhibitors in relation to type of resistance will be discussed here below.

Table 1. *Imatinib, nilotinib and dasatinib IC50 values for cellular proliferation assays. Preferred second-line agent in case of kinase domain mutants*

Type of mutation	Imatinib		Dasatinib		Nilotinib		First choice in second-line
	IC_{50} (nmol/l)	Fold change	IC_{50} (nmol/l)	Fold change	IC_{50} (nmol/l)	Fold change	
Wild type	260	1	13	1	0.8	1	Nilotinib or dasatinib
G250E	1,350	5	48	4	1.8	2	Nilotinib or dasatinib
Y253F	3,475	13	125	10	1.4	2	Dasatinib
Y253H	6,400	>25	450	35	1.3	2	Dasatinib
E255K	5,200	20	200	15	5.6	7	Dasatinib
E255V	6,400	>25	430	33	11	14	Dasatinib
T315I	6,400	>25	>2,000	>154	>200	>250	Experimental agents, Allo-SCT
M351T	880	3	15	1.2	1.1	1.4	Nilotinib or dasatinib
F359V	1,825	7	175	13	2.2	3	Dasatinib
H396P	850	3	41	3	0.6	0.8	Nilotinib or dasatinib
H396R	1,750	7	41	3	1.3	2	Nilotinib or dasatinib

Note: Fold change refers to the fold difference in the IC50, relative to wild type, which is set to 1.
Abbreviations: ns, no signal detected, Bcr-Abl not expressed

3. Treatment with second-line tyrosine kinase inhibitors

Dasatinib was the first second generation tyrosine kinase inhibitor that received accelerated U.S. Food and Drug Administration (FDA) approval in June 2006. Dasatinib is a potent dual BCR-ABL (325-fold greater potency than imatinib in vitro) and SRC family kinase inhibitor. First results of a phase I trial with dasatinib in 84 either imatinib-resistant or -intolerant patients appeared very promising.[32] Subsequently a phase II study was started and included 387 patients with chronic phase CML. With a median follow-up of approximately 15 months, a CHR and CCR was observed in 91% and 49%, respectively, of patients (Table 2) and PFS at 15 months was 90%.[33] The drug is generally well tolerated, common non-hematologic side effects include diarrhea, headache, fatigue, dyspnoe, pleural effusion, rash and nausea. Hematologic toxicity appeared very common. Side effects, especially pleural effusions, appeared less frequent with a dose of 100 mg qd as compared to dasatinib 70 mg bid, while response rated did not differ. Subsequently, dasatinib 100 mg qd became the recommended dose for chronic phase CML patients.

Nilotinib is a highly selective ABL inhibitor that received FDA approval in October 2007. Nilotinib is about 30-fold more potent than imatinib and appeared active in all phases of imatinib-resistant CML in a phase I trial.[34] Results of a phase 2 trial in 280 patient with imatinib-resistant or -intolerant CML were recently presented at the American Society of Hematology (ASH, 2008) after a median follow up of 15.5 months.[35,36] A MCR and CCR were observed in 58% and 42% of the patients, respectively, and OS estimated 91% at 18 months. Common non-hematologic side effects included mild skin rash, headache and nausea. Furthermore, hyperglycemia, hyperbilirubinemia and elevations in lipase were frequently observed biochemical abnormalities and regularly monitoring of these parameters is recommended. Rare adverse events included prolongation of the QT-interval (>500 milliseconds), fluid retention and pleural effusions.

Table 2. *Results of phase II studies of chronic phase CML patients with imatinib-resistance or -intolerance treated with second-line agents*

Type of response	Dasatinib IM-R	Dasatinib IM-I	Nilotinib IM-R	Nilotinib IM-I
	70 mg bid	70 mg bid	400 mg bid	400 mg bid
CHR	90%	94%	68%	90%
MCR	52%	80%	56%	63%
CCR	40%	75%	39%	50%
PFS	90%*		-	
OS	96%*		91%**	

Abbreviations: IM-R, imatinib-resistant; IM-I imatinib-intolerant; bid, twice daily; CHR, complete hematologic response; MCR, major cytogenetic response; CCR, complete cytogenetic response; PFS, progression-free survival; OS, overall survival.
*estimated at 15.5 months
**estimated at 18 months

Both drugs were also evaluated for efficacy and safety in previously untreated patients with chronic phase CML. Dasatinib was given at a dose of 100 mg qd or 50 mg bid in 50 patients with previously untreated chronic phase CML.37 A CCR and MMR were observed in 97% and 34% of the patients, respectively, at 1 year. Furthermore, 24 month EFS was 81%. Common toxicity grade 3-4 occurred infrequent, however, pleural effusion occurred in 21% of the evaluable patients and 27 patients (54%) required transient treatment interruption because of toxicity. Nilotinib was also evaluated in untreated patients and was studied in 49 patients with newly diagnosed CML.38 A CCR and MMR were observed in 96% and 52% of the patients, respectively, at 1 year. The estimated 2 year-EFS was 95%. Common toxicity grade 3 or 4 occurred infrequently, but 19 patients (36%) had transient treatment interruptions and 17 patients (32%) dose reductions due to toxicity. Collectively, early results with second-line agents appeared very promising. Complete cytogenetic responses were observed in > 90% and also some early CMR were noted in previously untreated chronic phase CML

patients. Follow-up, however, is still relatively short. Which inhibitor is nowadays preferred in second-line treatment of imatinib-resistant patients? The choice will primarily depend on the mechanisms of resistance involved. First, the most common mechanism of resistance against imatinib is a point mutations within the BCR-ABL kinase domain. The choice of second-line treatment will depend on the specific mutation detected, it's in vitro sensitivity to an alternative tyrosine kinase inhibitor, the size of the mutated clone, as well as the earlier cytogenetic and molecular response observed in a particular patient. The 7 most frequent occurring mutations (accounting for +/- 70% of mutations) are presented in Table 1 together with their in vitro inhibitory concentrations (IC-50) for the 3 tyrosine kinase inhibitors[39,40]. Furthermore, the preferred agent on the basis of in vitro sensitivity is indicated. For example the M244V, which is often detected in patients with chronic phase CML, appears sensitive for both nilotinib (IC50 38 nmol/L) and dasatinib (IC50 1.3 nmol/l). In contrast, the T315I mutation mutation confers resistance against all 3 tyrosine kinase inhibitors and patients with the T315I mutation should therefore be considered candidates for allo-SCT. If clonal evolution is considered responsible for resistance, malignant cell growth and proliferation may have become independent of BCR-ABL tyrosine kinase activity as outlined in the Introduction of this thesis. A broader agent as dasatinib may then be preferred in these patients and, unless a stable molecular response occurs, also consolidation by allo-SCT should be considered then. Apart from taking the type of mutation and cytogenetics into account, also the side-effects of the alternative tyrosine kinase inhibitor should be weighed. This is especially evident in patients with imatinib intolerance. Dastinib and nilotinib are both effective as alternative treatment in case of imatinib-intolerance as illustrated in Table 1. Of note, no significant cross-intolerance between dasatinib and imatinib was observed for non-hematologic toxicities. However, patients who discontinued imatinib for hematologic toxicity frequently experienced similar toxicity with either dasatinib or nilotinib. In case of severe fluid retention with imatinib, nilotinib may be preferred as second-line tyrosine kinase inhibitor in order to prevent pleural effusion. Patients with a history of diabetes, pancreatitis, or cardiovascular disease should refrain from nilotinib, because nilotinib can provoke these conditions. Collectively, a careful history, physical examination and laboratory investigation is recommended in patients with intolerance to imatinib and all aspects should be weighed before choosing a second-line tyrosine kinase inhibitor in a particular patient. It should be emphasized that imatinib is still first choice in newly diagnosed CML. A direct comparison of imatinib with nilotinib or dasatinib with sufficient follow-up is obviously required to establish if one of the new tyrosine kinase inhibitors is to be preferred in terms of efficacy as well tolerability. Randomized phase III trials with nilotinib or dasatinib versus imatinib are currently underway, but it may take several years before mature results are available.

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9

SUMMARY
SAMENVATTING

English Summary

Chronic myeloid leukemia (CML) is a rare disease worldwide and is characterized by a unique reciprocal translocation between the long arms of chromosomes 9 and 22 resulting in an aberrant chromosome 22, also known as the Philadelphia chromosome. The introduction of imatinib and accelerated Food and Drug Administration (FDA) approval in 2001 has revolutionized treatment of patients with CML. Response to treatment in chronic phase CML nowadays is excellent with an overall survival of approximately 80-90% at 6-7 years from start of tyrosine kinase inhibitor therapy. However, some patients don't respond favourably and primary and secondary resistance to imatinib is of concern. Different mechanisms of resistance against imatinib have been demonstrated including mutations in the *BCR-ABL* kinase domain, BCR-ABL gene amplification and overexpression, clonal evolution, multidrug resistance, and other mechanisms affecting metabolism of imatinib. One of the strategies to prevent resistance is combination therapy by imatinib and cytarabine. The main question in this thesis was to address whether standard or high-dose chemotherapy with cytarabine would improve the response rate and overall outcome in first chronic phase CML.

Following a review in Chapter 1 addressing the different mechanisms of resistance, which can currently be identified, we first studied whether intensified cytarabine followed by interferon alfa (IFN-α) would be superior to low-dose cytarabine combined with IFN-α. Interferon alfa had been the cornerstone of therapy before the introduction of tyrosine kinase inhibitor therapy. The addition of low-dose cytarabine, given subcutaneously at a dose of 20 mg/m^2 per day had been shown to improve the cytogenetic response rate and overall outcome in a large study performed in the nineties in France. Following that study, the Dutch-Belgian cooperative group HOVON performed a prospective phase III study in newly diagnosed patients with CML (HOVON-38), which accrued 118 patients. More specifically, the question was addressed whether intensified cytarabine followed by IFN-α would be superior to low-dose cytarabine combined with IFN-α. Early cytogenetic response rates appeared higher with intensified cytarabine, but most responses were rapidly lost and event-free survival proved similar between the 2 treatment arms, despite IFN-α maintenance therapy. Therefore, it was concluded that high-dose cytarabine based chemotherapy followed by IFN-maintenance cannot be preferred over the combination of low-dose cytarabine and IFN-α in first chronic phase CML patients.

Prospective studies evaluating imatinib for patients with first chronic phase CML started around the turn of the century worldwide. Given early reports of observed resistance, the HOVON cooperative group set to evaluate novel approaches to prevent resistance and improve response rates. In vitro studies showed that imatinib can be combined with several cytotoxic agents, but the combination of imatinib and cytarabine was suggested to act synergistically both against BCR-ABL positive cell lines and primary cells from CML patients. Therefore,

HOVON set out to explore the feasibility and efficacy of combination therapy of 4 different dosages of imatinib (200 mg, 400 mg, 600 mg or 800 mg) and 2 different dosages of cytarabine (200 mg/m^2 or 1000 mg/m^2) intravenously, according to predefined successive dose levels (HOVON-51). The feasibility results of that combination are presented and discussed in Chapter 3. All dose levels proved feasible. High-dose imatinib (600 mg or 800 mg) extended the time to platelet recovery as compared to a standard-dose (400 mg). Intermediate-dose cytarabine (1000 mg/m^2) prolonged time to neutrophil recovery and platelet recovery compared with a standard-dose (200 mg/m^2). Furthermore, a higher dose of cytarabine was associated with more infectious complications. Therefore, the combination of a standard-dose of intravenous cytarabine with different dosages of imatinib may be preferred, because it was tolerated very well and could also be given on an outpatient basis.

Early results detailing response are also presented in Chapter 3, but an in-depth analysis of efficacy with an extended median follow-up of 55 months, is described in Chapter 4. Efficacy end points included cumulative incidences of response at the hematologic, cytogenetic and molecular levels; survival end points including overall, progression-free, and event-free survival; incidences of resistance, including an evaluation of the mutations occurring in patients without a major molecular response (MMR). The 5-year cumulative incidences of a complete cytogenetic response (CCR) and complete molecular response (CMR) were 89% and 53%, respectively. A higher Sokal risk score was inversely associated with CCR. A higher dose of imatinib and a higher dose of cytarabine were both associated with increased CMR. Progression-free survival and overall survival at 5 years were 92% and 96%, respectively. Achieving a MMR at 1 year was associated with complete absence of progression and a probability of achieving a CMR of 89%. In conclusion, following earlier in vitro findings, our clinical results seem to suggest an additive or even synergistic effect of cytarabine as was observed in vitro. However, only a prospective phase III study can more reliably assess the contributory role of cytarabine to imatinib. As a result, a prospective randomized trial was designed, which is currently being performed (HOVON-78).

Although the molecular response rate was high, especially in patients receiving a higher dose of imatinib, still approximately 40% of those patients did not obtain a CMR. Persistent residual disease at the molecular level may be designated as molecular resistance and has been suggested to be accounted for by quiescent malignant stem cells. P-glycoprotein (P-gp), which is encoded by the ABCB1 multi drug resistance (MDR1) gene, has been demonstrated to mediate efflux of imatinib and is efficiently expressed by both normal and malignant hematopoietic progenitor cells. In Chapter 5 we set out to evaluate whether the 3 most common single nucleotide polymorphisms (SNPs) of the ABCB1 gene (C1236T; G2677T/A; C3435T) would be associated with molecular response and resistance. Results showed that the probability of developing either a major or complete molecular response strongly depended on SNP-genotype. Specifically the TT genotype of all 3 SNPs appeared associated with molecular resistance, which may in be explained by the 2677TT genotype in particular

as the 2677TT genotype is associated with enhanced efflux and higher P-gp activity in vitro. Collectively, these results suggest a role for P-gp mediated drug efflux in malignant hematopoietic stem cells, which account for persistent residual disease.

In Chapter 6 a new flowcytometric approach to identify CML stem cell is described. Chronic myeloid leukemia stem cells could be discriminated from normal stem cells by higher CD34 and CD45 expression and different forward/sideward light scatter properties in newly diagnosed CML patients. In addition, expression of other lineage markers as CD7, CD11b and CD56, which were not expressed on normal stem cells, helped to distinguish malignant from benign stem cells. Using fluorescence in situ hybridization (FISH) on FACS sorted cells, it was shown that sorted populations with aberrant expression patterns were indeed *BCR-ABL* positive. Long term culture of sorted stem cell subsets with subsequent FISH analysis further proved their malignant stem cell character. It was concluded that flowcytometry is a powerful tool to discriminate malignant and normal stem cells in CML. This new technique may thereby expand our possibilities to identify new CML stem cell specific targets and may improve efficacy assessment of CML treatment.

Recently, 2 new Abl-tyrosine kinase inhibitors, dasatinib and nilotinib, were approved for second-line treatment of CML patients with intolerance or resistance to imatinib. In Chapter 7, we describe a patient with a NUP214-ABL1-positive T-cell acute lymphoblastic leukemia (T-ALL), who was treated with dasatinib monotherapy prior to combination chemotherapy and continued dasatinib. The patient achieved a strikingly rapid complete hematologic and cytogenetic remission already after 3 weeks of dasatinib monotherapy. In addition, the patient obtained a complete immunological and molecular remission upon completion of induction chemotherapy in combination with dasatinib. Following an earlier report with disappointing results with imatinib, this observation clearly shows the clinical efficacy of dasatinib in NUP214-ABL1-positive T-cell ALL. Dasatinib may exert a more stronger apoptotic effect as a result of it's ability to inhibit several other kinases apart from ABL1, that may play a role in T-cell proliferation. Collectively, it is suggested that dasatinib may be preferred as an inhibitor in NUP214-ABL1-positive T-ALL patients.

Finally, the results of the studies presented are interrelated in Chapter 8 (General discussion) and 3 subjects are addressed in more detail, including the role of cytarabine, the current place of molecular diagnostic techniques, and the development of the second-generation tyrosine kinase inhibitors.

Nederlandse Samenvatting

Chronische myeloïde leukemie (CML) is een weinig voorkomende vorm van leukemie, die wordt gekenmerkt door het zogenaamde "Philadelphia" chromosoom, dat in de leukemiecellen kan worden aangetroffen. Dit afwijkende chromosoom (nummer 22) ontstaat door een wederkerige translocatie tussen de lange armen van de chromosomen 9 en 22. Als gevolg van deze translocatie ontstaat er een kankergen, dat codeert voor een enzym (BCR-ABL) met verhoogde kinase activiteit, waarbij eiwitten met een tyrosine in de peptidenvolgorde gefosforyleerd worden en daarmee aan activiteit kunnen winnen. De kinase activiteit van dit enzym bleek krachtig geremd te kunnen worden door een speciaal daartoe ontworpen medicament, imatinib, dat specifiek bindt aan het enzym ABL. Imatinib is een betrekkelijk jong medicament, waarmee klinisch onderzoek in 1998 startte, hetgeen al gauw gevolgd werd door goedkeuring door de Amerikaanse en Europese instanties in 2001. Imatinib heeft een ommekeer teweeggebracht in de behandeling van patiënten met CML. De resultaten met imatinib bij patiënten met CML in chronische fase bleken uitstekend, waarbij de geschatte overleving momenteel rond de 80-90% is op 6-7 jaar na starten van de behandeling. Er zijn echter patiënten die minder goed op de behandeling reageren en direct (primair) resistent zijn, of na een initiële respons deze respons verliezen en in 2e instantie (secundair) resistent blijken tegen imatinib. Deze resistentie tegen imatinib is een belangrijk onderzoeksterrein geworden. Er zijn verschillende mechanismen ontdekt, die resistentie tegen imatinib kunnen veroorzaken. Belangrijke mechanismen zijn onder meer: mutaties in het BCR-ABL kinase domein; BCR-ABL gen amplificatie en overexpressie; klonale evolutie; multidrug resistentie; en andere mechanismen die het metabolisme van imatinib beïnvloeden. Een van de strategieën om resistentie tegen imatinib te voorkomen is combinatietherapie, waarbij imatinib gecombineerd wordt met een medicament met eerder bewezen activiteit tegen CML, zoals cytarabine, een celdodend chemotherapeuticum (cytostaticum). De belangrijkste vraag van dit proefschrift was of de toevoeging van cytarabine aan imatinib, de behandelingsresultaten verbetert bij patiënten met CML in de chronische fase.

In het 1e hoofdstuk worden verschillende mechanismen van resistentie besproken vooral in het licht van het belang in de kliniek. In het 2e hoofdstuk wordt een stapje terug in de tijd gedaan en wordt een studie beschreven met 2 medicamenten (interferon alfa en cytarabine), die voor de introductie van imatinib gebruikt werden bij de behandeling van CML. Interferon alfa (IFN-α) was de hoeksteen van de behandeling van patiënten met CML voor de introductie van imatinib. Eerder onderzoek in Frankrijk had al uitgewezen dat toevoegen van een lage dosering cytarabine de behandelingsresultaten verbetert ten opzichte van een behandeling met alleen IFN-α. Geïnspireerd door deze studie en de goede resultaten van cytarabine in acute leukemie ontwierpen Nederlandse en Belgische hematologen verenigd in HOVON (Stichting Hemato-Oncologie Volwassenen Nederland) een prospectieve, 2-armige, fase III studie, waarin deze combinatiebehandeling werd vergeleken met een

experimentele behandeling bestaande uit intensieve chemotherapie met cytarabine, gevolgd door IFN-α onderhoudsbehandeling: de HOVON-38 studie. Na verkregen toestemming werden 118 patiënten in deze studie opgenomen. Vervolgens kregen na loting 32 patiënten de standaardbehandeling en 28 patiënten kregen de experimentele behandeling. De studievraag was of intensieve chemotherapie met cytarabine gevolgd door IFN-α beter zou zijn dan een lage dosering cytarabine gecombineerd met IFN-α. De resultaten werden uitgedrukt in afname van het percentage Philadelphia-chromosoom positieve metafasen (de zogenaamde cytogenetische respons) en in overlevingsparameters. Weliswaar bleek intensieve chemotherapie een hoger responspercentage op te leveren, maar deze respons bleek van korte duur en de overlevingsparameters bleken niet wezenlijk verschillend tussen de beide studiearmen. De conclusie was dan ook dat chemotherapie met hoge dosis cytarabine gevolgd door IFN-α onderhoudsbehandeling niet tot betere resultaten leidt dan combinatietherapie bestaande uit een lage dosering cytarabine met IFN-α bij patiënten met CML in de chronische fase.

Prospectieve studies met imatinib bij patiënten met CML zijn wereldwijd van start gegaan rond de eeuwwisseling. Naar aanleiding van vroege gegevens over resistentieontwikkeling tegen imatinib besloot HOVON een nieuwe benadering te onderzoeken met het doel zowel de respons als de uiteindelijke overleving bij deze patiënten te verbeteren. In het laboratorium was al aangetoond dat imatinib effectief gecombineerd kon worden met een aantal celdodende medicamenten, waarbij de combinatie van imatinib met cytarabine de beste resultaten leek op te leveren. De HOVON-51 studie werd ontwikkeld om de combinatie van verschillende doseringen imatinib (200 mg, 400 mg, 600 mg of 800 mg) met cytarabine (200 mg/m^2 of 1000 mg/m^2, dagelijks gedurende 1 week intraveneus gegeven) te onderzoeken. Deze studie had een zogenaamd haalbaarheidskarakter, waarbij opeenvolgende groepjes patiënten een steeds wat hogere dosis combinatietherapie kregen, als het voorafgaande dosisniveau veilig was gebleken. De resultaten van deze studie (HOVON-51) worden in hoofdstuk 3 beschreven. Alle dosiscombinaties bleken goed haalbaar. Cytarabine veroorzaakte een tijdelijk tekort aan bloedplaatjes en aan witte bloedcellen (leukocyten). Het herstel van zowel het aantal bloedplaatjes als leukocyten bleek significant langer te duren na de intermediaire dosering cytarabine (1000 mg/m^2) dan na de standaard dosering (200 mg/m^2). Ook een hogere dosering imatinib (600 mg of 800 mg vergeleken met 400 mg) ging gepaard met een langer durend tekort aan bloedplaatjes. Infecties werden vaker gezien na de hogere dosering cytarabine dan na de standaard dosering cytarabine. Op grond van deze gegevens werd geconcludeerd dat combinatietherapie goed haalbaar is, maar dat de combinatie van een standaard dosering cytarabine wellicht de voorkeur zou moeten genieten, gezien de verdraagbaarheid, de poliklinische toepasbaarheid, alsmede de uitstekende initiële resultaten.

De initiële resultaten wat betreft respons op combinatietherapie (HOVON-51) staan vermeld in hoofdstuk 3, maar rijpere gegevens, na de patiënten gedurende langere tijd gecontroleerd te hebben, worden beschreven in hoofdstuk 4. Eindpunten van de in

hoofdstuk 4 beschreven effectiviteitanalyse zijn onder meer de cytogenetische en moleculaire responspercentages; overlevingsparameters; en voorkomen van resistentie. Op 5 jaar na start behandeling bleek 89% van de patiënten een complete cytogenetische respons (CCR) te hebben ontwikkeld en 53% zelfs een complete moleculaire respons (CMR). Bij een CMR kunnen met heel gevoelig moleculair onderzoek naar het BCR-ABL kankergen geen tekenen van de ziekte meer worden aangetoond in het beenmerg. De gevoeligheid van dit onderzoek laat toe, dat 1 leukemiecel op 100.000 normale cellen nog aangetoond kan worden, maar dat een lagere frequentie van leukemie niet meer opgepikt wordt. De 5-jaars overleving bleek met 96% uitstekend en de overleving op 5-jaar zonder progressie naar een gevorderd stadium (acceleratiefase of blastencrisis) de zogenaamde progressievrije overleving was 92%. Patiënten, die een hogere dosering kregen van imatinib dan wel van cytarabine, toonden betere resultaten. Met name de patiënten, die een moleculaire respons bereikten, lieten uitzonderlijke resultaten zien met volledig uitblijven van ziekteprogressie. Concluderend kan gezegd worden dat onze resultaten duidelijk suggereren, dat cytarabine bijdraagt aan de effectiviteit van imatinib alleen, daar met imatinib monotherapie niet eerder dergelijke snelle, hoge moleculaire responspercentages werden gerapporteerd. Echter, alleen door middel van een prospectieve fase III studie, waarin geloot wordt tussen de combinatiebehandeling en imatinib, kan de ware bijdrage van cytarabine aan imatinib vastgesteld worden. Met dit doel is de HOVON-78 ontwikkeld, die meer definitief de vraag moet beantwoorden of de combinatie van imatinib (800 mg) met een standaard dosering cytarabine (200 mg/m^2) effectiever is dan imatinib (800 mg) monotherapie.

Hoewel de moleculaire respons in de HOVON-51 studie hoog was, bereikten ongeveer 40-50% van de patiënten geen CMR. Persisterende restziekte op moleculair niveau wordt ook wel moleculaire resistentie genoemd en eerder is gesuggereerd dat deze veroorzaakt zou kunnen worden door niet-actieve, 'quiescent', CML stamcellen. Stamcellen brengen het eiwit P-glycoproteïne (P-gp) tot expressie, welk eiwit bepaalde medicamenten, waaronder ook imatinib actief de cel uit kan pompen. Ook CML stamcellen brengen P-gp tot expressie. P-glycoproteïne wordt gecodeerd door het ABCB1 multidrug resistentie (MDR1) gen, waarvan een aantal varianten bekend zijn. Deze varianten verschillen in slechts 1 bouwsteen (nucleotide) van de DNA-volgorde en worden daarom "single nucleotide polymorphisms" (SNPs) genoemd. In hoofdstuk 5 wordt het onderzoek beschreven naar de 3 meest voorkomende SNPs van het ABCB1 gen (C1236T; G2677T/A; C3435T), waarbij onderzocht werd of deze SNPs geassocieerd waren met moleculaire resistentie. De kans op het ontwikkelen van een moleculaire respons bleek sterk afhankelijk van het SNP genotype. Vooral het TT genotype van alle 3 de SNPs bleken geassocieerd met moleculaire resistentie, welke waarschijnlijk het best verklaard kan worden met het 2677TT genotype, daar dit genotype geassocieerd is met verhoogde pompactiviteit van P-gp. Samenvattend suggereren deze resultaten een duidelijke rol voor P-gp in de moleculaire resistentie zoals gevonden bij CML patiënten, die een hogere dosis imatinib kregen.

In hoofdstuk 6 wordt een nieuwe methode beschreven om in het laboratorium onderscheid te kunnen maken tussen normale stamcellen en Philadelphia-positieve of maligne CML stamcellen. Deze methode maakt gebruik van antistoffen, die oppervlakte-eiwitten herkennen, welke verschillend tot expressie komen bij normale en CML stamcellen en vervolgens met een flowcytometer zichtbaar kunnen worden gemaakt. Het bleek dat CML stamcellen zich onderscheiden van normale stamcellen door een hogere CD34 en CD45 expressie en een andere wijze van lichtverstrooiing. Bovendien bleken deze maligne stamcellen ook andere merkereiwitten zoals CD7, CD11b en CD56 tot expressie te brengen. Deze komen eigenlijk niet voor op normale stamcellen en bleken nuttig bij de differentiatie ten opzichte van normale stamcellen. Na selectie van verschillende stamcelpopulaties op basis van de gevonden expressiepatronen, bleek dat de populaties met aberrante expressie ook voornamelijk cellen met het Philadelphia chromosoom bevatten. Vervolgens werden geselecteerde populaties ook gekweekt, waarna chromosoom analyse aanvullend bewijs leverde voor het maligne stamcelkarakter. Samenvattend kan geconcludeerd worden dat analyse door middel van een beperkt panel van antistoffen het mogelijk maakt om met behulp van de flowcytometer maligne en normale stamcellen van elkaar te onderscheiden bij CML patiënten. In de toekomst kan deze nieuwe techniek behulpzaam zijn om nog nauwkeuriger behandelingsresultaten te meten en zou eventueel ook kunnen bijdragen aan nieuwe behandelingsstrategieën, die specifiek op CML stamcellen gericht gaan worden.

Zeer recent werden de 2 nieuwe ABL kinase remmers dasatinib en nilotinib goedgekeurd voor gebruik bij CML patiënten, die intolerant of resistent zijn tegen imatinib. In hoofdstuk 7 beschrijven we de ziektegeschiedenis van een patiënt met een T-cel acute lymfoblasten leukemie (T-ALL), waarbij in de T-cellen het kankergen NUP214-ABL1 werd aangetoond. Onderzoek elders had eerder al uitgewezen dat het ABL kinase ook bij deze leukemie een oorzakelijke rol speelt en dat remming van het kinase een nieuwe behandelingsmogelijkheid biedt. Echter, de resultaten met imatinib bleken teleurstellend, om welke reden wij bij onze patiënt de behandeling startten met dasatinib. Na een korte periode van behandeling met alleen dasatinib, bereikte de patiënt al binnen 3 weken een complete hematologische en cytogenetische respons. Daarna werd doorgegaan met een combinatie van dasatinib en de gebruikelijke chemotherapie, zoals die bij acute lymfatische leukemie doorgaans gegeven wordt. Er werd een complete immunologische en ook moleculaire remissie bereikt na afsluiten van deze combinatietherapie. Al met al laat deze observatie duidelijk effectiviteit zien van dasatinib bij deze bijzondere vorm van acute leukemie. De verklaring naar de mogelijk betere werkzaamheid van dasatinib ten opzichte van imatinib is mogelijk gelegen in het sterkere celdodende effect van dasatinib alsmede de mogelijkheid om met dasatinib behalve ABL ook andere kinases te remmen, die een rol spelen in de maligne T-cellen. Samenvattend suggereert deze observatie dat dasatinib wellicht de kinase remmer van voorkeur zou moeten zijn bij deze vorm van acute lymfatische leukemie.

Tenslotte worden in het laatste hoofdstuk de resultaten uit dit proefschrift in meer algemene zin besproken, waarbij 3 onderwerpen nader onder de loep worden genomen. Met name wordt stilgestaan bij de rol van cytarabine in de huidige CML behandeling, de toepassing daarbij van moleculaire diagnostiek, alsmede de zeer recente ontwikkeling van de 2e generatie kinase remmers.

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Curriculum vitae

Wendy Deenik werd geboren op 17 januari 1971 te Fort Portal (Uganda). In 1989 behaalde zij het VWO-diploma aan het Wagenings Lyceum. In hetzelfde jaar werd gestart met de studie geneeskunde aan de Universiteit van Amsterdam. In 1992 deed zij keuze onderzoek in het Antoni van Leeuwenhoek ziekenhuis (begeleiders: Prof. Dr. W.J. Mooi en Prof. Dr. B.B.R. Kroon), waar zowel haar wetenschappelijke belangstelling als interesse voor de oncologie werd gewekt. Zij behaalde haar artsexamen cum laude in februari 1996, waarna zij als AGNIO interne geneeskunde in het St. Lucas Andreas ziekenhuis werkte. In september 1996 werd met de opleiding interne geneeskunde gestart, waarvan de eerste 3 jaar in ditzelfde ziekenhuis (opleider: Dr. E. Monasch). De opleiding tot internist werd in september 1999 voortgezet in het VU medisch centrum te Amsterdam (opleider: Prof. Dr. J. van der Meer), waarna registratie in september 2002 volgde. In het laatste jaar van de opleiding werd het aandachtsgebied hematologie gevolgd (opleider: Prof. Dr. P.C. Huijgens), waarna registratie volgde in november 2003. Vanaf oktober 2003 tot oktober 2008 was zij als internist-hematoloog verbonden aan het Erasmus medisch centrum (hoofd: Prof. Dr. B. Löwenberg). Tijdens deze periode werd dit proefschrift geschreven (promotor: Prof. Dr. J.J. Cornelissen). Eind 2008 is zij korte tijd werkzaam geweest op de afdeling interne oncologie in het Erasmus medisch centrum. Vanaf 1 april 2009 is zij werkzaam als internist-hematoloog op beide locaties (Blaricum en Hilversum) van Tergooiziekenhuizen. Zij heeft een zoon, Kevin.