New treatment strategies in myelodysplastic syndromes and acute myeloid leukemia

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Summary, discussion and future perspectives
SUMMARY

The “3+7 regimen” that is based on a combination of an anthracyclin and cytarabine is since more than 40 years the backbone for the treatment of AML. This regimen has cured a considerable number of patients. However, the results in older patients are disappointing, also when post-remission maintenance therapy was added to the regimen.\textsuperscript{1,2} The unfavorable results at older age are partially related to increased comorbidity. However, more dominant are unfavorable disease characteristics, such as adverse cytogenetic abnormalities, high rates of MDS-related AML and therapy-related AML, high incidence of multidrug resistance and distinct gene expression profiles in older patients.\textsuperscript{3-5} Therefore, new therapies that more efficiently target malignant cells and are also applicable in less fit patients, are desired. In this thesis, two new treatment strategies were studied in a clinically relevant setting and \textit{in vitro}, respectively.

With the emergence of the hypomethylating agents (HMAs) azacitidine and decitabine, a new less intensive treatment strategy became available for higher-risk MDS and AML patients. A survival benefit of azacitidine compared to conventional care (including intensive chemotherapy, best supportive care (BSC), or low-dose cytarabine) was demonstrated in 2009 in a phase III randomized controlled trial in higher-risk MDS en AML patients with less than 30% bone marrow blasts.\textsuperscript{6} However, little was known about the efficacy of azacitidine in daily clinical practice, without strict inclusion and exclusion criteria, and in AML patients with more than 30% bone marrow blasts. In Chapter 2, we analyzed the treatment results of a cohort of 90 MDS, CMML, and AML patients who participated in the Dutch compassionate use named patient program. In this program, patients could receive azacitidine before registration in the Netherlands, i.e. before December 2008. Results revealed overall response rates (complete- or partial remission (CR, PR) and hematologic improvement (HI)) of about 50% with CR/PR rates of 26%, which was comparable to the AZA-001 trial. Median overall survival (OS) tended to be shorter with 13.0 months (range 9.8-16.2), but was comparable to the OS in the French named patient program of 13.5 months. In our cohort, several previously described predictors for poor outcome could be confirmed, including poor-risk cytogenetics, circulating blasts, and poor WHO performance score. Interestingly, a significantly longer OS was observed in the small group of patients (16%) who had an early platelet response, defined as platelet count doubling after the first cycle (four weeks) of azacitidine.

In the extended named patient program, also patients with a bone marrow blast count over 30% could be treated with azacitidine. In Chapter 3, the treatment results of these patients were analyzed and compared with AML patients with 20%-30% blasts. Results revealed no differences in response rates and OS in patients with blast counts higher or lower than 30%. More predictive for disadvantageous outcome than the percentage of bone marrow blasts, were lack of response to azacitidine, poor cytogenetic risk, poor WHO performance score, therapy-related AML, and white blood cell (WBC) count over 15 x 10\textsuperscript{9}/L. The efficacy of azacitidine in AML patients with more than 30% blasts has recently been confirmed in a large phase III trial.\textsuperscript{7}
In our center, a relatively large population of older AML patients has been treated with azacitidine. In Chapter 4, we analyzed the characteristics and outcome of all consecutive AML patients of 60 years and older diagnosed and treated in the University Medical Center Groningen between 2003 and 2010. We compared patients receiving azacitidine with patients receiving intensive chemotherapy or BSC. A significantly improved OS was observed in the azacitidine-treated patients compared to the BSC group, which was partly related to the therapy and partly to more favorable patient- and disease characteristics in the azacitidine group. Interestingly, azacitidine and intensive chemotherapy resulted in similar OS. Also after correction for baseline characteristics and known risk factors, OS did not differ between both groups. Because the follow-up time and number of azacitidine-treated patients in this study were relatively limited, we reassessed our cohort in Chapter 5 after a median follow-up time of four years and with extended inclusion of older AML patients until August 2015. Compared to patients receiving BSC only, azacitidine-treated patients showed a significant survival advantage. The survival of azacitidine-treated patients after the longer follow-up time was still comparable with patients receiving intensive chemotherapy, unless the latter had acute promyelocytic leukemia or subsequently underwent allogeneic hematopoietic cell transplantation (allo-HCT).

Mutations in the tumor suppressor gene TP53 are frequently associated with complex karyotypes, poor response to intensive chemotherapy (about 28% CR in TP53-mutated patients versus 50% CR in other adults with complex karyotype AML), and shorter OS and relapse-free survival after CR. An important question is whether these patients can benefit from an alternative type of therapy, i.e. HMAS. In Chapter 5, we assessed bone marrow samples of 47 older AML patients treated with HMAS by using immunohistochemistry to identify TP53 overexpression in AML cells, which is indicative for TP53 mutation. OS of the 22 patients with TP53 overexpression was shorter compared to patients with normal TP53 expression. Nonetheless, response rates did not significantly differ and both patient groups had improved survival when achieving a response to azacitidine. Further, achieving CR in two patients with baseline TP53 overexpression was associated with disappearance of TP53++ cells, indicating that TP53-mutated AML cells can be targeted by HMAS. These data suggest that HMAS may be beneficial in older AML patients with a TP53 mutation.

Understanding the tumor biology of AML and differences between AML stem cells and normal hematopoietic stem cells can lead to new therapeutic strategies. One of the observed differences between AML (stem) cells and normal hematopoietic stem cells concerns the ubiquitin-proteasome system. Increased proteasome expression, proteasome activity, and NF-κB activity have been described in AML stem cell-enriched cell populations, suggesting that the proteasome is a potential druggable target. The first-generation proteasome inhibitor bortezomib reduced NF-κB activity and induced apoptosis, especially in more mature CD34+ AML cell populations, whereas the more primitive AML CD34+ cells were less sensitive. In Chapter 6, we investigated whether the second-generation proteasome inhibitors carfilzomib and oprozomib had improved efficacy to target patient-derived primitive AML cells compared to bortezomib. We observed a larger reduction of AML stem cell frequencies in long-term cultures after incubation with carfilzomib as
compared to bortezomib. Also quiescent AML CD34^+CD38^- cell percentages were more reduced after incubation with carfilzomib. A higher efficacy of carfilzomib could be related to its irreversible binding to the proteasome, resulting in prolonged proteasome inhibition, whereas shorter-lived effects can be expected of bortezomib that binds reversibly. Indeed, we observed reduction of proteasome activity for a longer period after incubation with carfilzomib compared to bortezomib. Importantly, normal CD34^+ cells were less affected, which could be due to lower normal proteasome abundance and proteasome activity, associated with lower dependency on the proteasome. In AML CD34^+ cells, proteasome activity indeed tended to be increased. Further, 9 out of 17 proteasome subunit-coding genes were significantly higher expressed in AML CD34^+ cells compared to normal CD34^+ cells. Previous studies have indicated that upregulation of anti-apoptotic MCL-1 inhibited the apoptotic effect of bortezomib in AML CD34^+ cells. Likewise, we observed upregulation of MCL-1 upon incubation with carfilzomib and oprozomib. Co-treatment with the pan-Bcl-2 inhibitor obatoclax, which also inhibits MCL-1, enhanced the apoptotic effects of the proteasome inhibitors on primitive AML CD34^+ cells, suggesting that carfilzomib in conjunction with an apoptotic drug might be a potential new treatment strategy in AML patients.
DISCUSSION AND FUTURE PERSPECTIVES

Treatment of older patients with higher-risk MDS or AML

Treatment of older AML- or higher-risk MDS patients is clinically challenging. With a life expectancy of approximately 20 years at 65 years of age\textsuperscript{10}, there is much to gain by effective therapy. Different studies have demonstrated that treatment with either intensive or non-intensive therapy results in better survival and quality of life than BSC.\textsuperscript{11,12} Results of the Swedish Acute Leukemia registry indicate that most older AML patients still benefit from intensive chemotherapy, since OS was better in regions where more patients received intensive therapy.\textsuperscript{12} Besides, a phase II clinical trial and post-hoc analyses of phase III trials indicate that allo-HCT following reduced-intensity conditioning is feasible and associated with better overall survival and relapse-free survival in AML patients between 60 and 75 years of age, especially in those with intermediate and adverse risk AML.\textsuperscript{13-15}

However, many MDS and AML patients are not fit enough for standard intensive induction therapy and for many older patients with adverse disease characteristics it is questionable whether the expected treatment benefit outweighs the treatment-related side effects and mortality risk. Especially in these patients, less-intensive treatment strategies are essential. Less-intensive therapy with the hypomethylating agent azacitidine has become available for higher-risk MDS and AML patients after a survival benefit has been reported in randomized phase III studies in 2009 and 2015 (see indications in Table 1).\textsuperscript{6,7,16} As AML and MDS patients included in randomized clinical trials tend to be younger, have better performance scores, less comorbidities, and less adverse disease characteristics compared to patients not included in trials\textsuperscript{17}, caution should be taken to extrapolate trial data to the general population of MDS and AML patients. In this thesis, we studied the use of azacitidine in MDS, CMML, and AML patients in daily clinical practice. Our data from the Dutch named patient program confirm the effectivity of azacitidine in clinical practice with responses in about half of the patients and a median OS of 13.0 months. Also AML patients with more than 30% bone marrow blasts showed favorable responses to azacitidine in our extended named patient program; they had similar response and survival rates as patients with less than 30% blasts. Recently, the AZA-AML-001 trial confirmed the efficacy of azacitidine in AML patients (age ≥65 years) with more than 30% bone marrow blasts. In this trial, median OS of AML patients treated with azacitidine was 10.4 months versus 6.5 months with conventional care (i.e. preselected low-dose cytarabine, intensive chemotherapy, or BSC). This difference did not reach statistical significance in the primary analysis (p=0.1009), but a pre-specified analysis censoring patients from the moment they received subsequent therapy after study drug discontinuation indicated a significant survival benefit of 5.2 months for azacitidine. Further, analyses of the preselected subgroups revealed a significant survival benefit for azacitidine compared to BSC (median OS 5.8 versus 3.7 months; HR 0.60 (95% CI 0.38-0.95), p = 0.029), a trend towards better OS compared to low-dose cytarabine (median OS 11.2 versus 6.4 months;
HR 0.90 (95% CI 0.70-1.16), p = 0.43), and a similar median OS of azacitidine and intensive chemotherapy (13.3 versus 12.2 months; HR 0.89 (95% CI 0.52-1.43), p = 0.50). Based on these results, azacitidine was approved by the European Medicine Agency in October 2015 for the treatment of AML patients of 65 years and older with more than 30% blasts and ineligible for allo-HCT (Table 1). Likewise, a marginally significant survival benefit was observed in older AML patients treated with decitabine (20 mg/m² in 1 hour for 5 days/month) compared to low-dose cytarabine or BSC (7.7 versus 5.0 months, HR 0.82 (95% CI 0.68-0.99), p = 0.037 at secondary analysis), which has led to approval of decitabine in 2012 for AML patients of 65 years and older unfit for intensive chemotherapy (Table 1).

Table 1. Approved indications for HMAs by the European Medicine Agency

<table>
<thead>
<tr>
<th></th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azacitidine 75 mg/m² s.c. 7/28d</td>
<td>Adults who are not eligible for hematopoietic stem cell transplantation with:</td>
</tr>
<tr>
<td></td>
<td>• MDS with IPSS intermediate-2 or high risk</td>
</tr>
<tr>
<td></td>
<td>• CMML with 10–29% bone marrow blasts without myeloproliferative disorder</td>
</tr>
<tr>
<td></td>
<td>• AML with 20–30% blasts and multi-lineage dysplasia, according to WHO</td>
</tr>
<tr>
<td></td>
<td>classification</td>
</tr>
<tr>
<td></td>
<td>• AML with &gt; 30% bone marrow blasts and age ≥ 65 years</td>
</tr>
<tr>
<td>Decitabine 20 mg/m² i.v. 5/28d</td>
<td>Adults aged ≥ 65 years who are not eligible for standard induction therapy with:</td>
</tr>
<tr>
<td></td>
<td>• AML, newly-diagnosed de novo or secondary</td>
</tr>
</tbody>
</table>

IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; WHO, World Health Organization.

Side-effects of azacitidine and decitabine are relatively mild in MDS as well as in older AML patients. They include hematologic toxicity (including grade 3-4 cytopenias), most often resolving after the first two cycles, gastrointestinal side effects (especially for azacitidine) that are usually controllable with simple measures, and injection site reactions in case of azacitidine. Tolerability of HMAs is generally much better in older patients compared to intensive chemotherapy, which was reflected in our AML cohort by a lower number of days in the hospital, lower transfusion requirements, and a limited drop-out due to drug toxicity. An additional advantage of HMAs is the administration in an outpatient setting. Development of oral azacitidine (CC-486), at present assessed in phase I studies in low-risk MDS and AML patients, may further enhance applicability in older and frail patients. In summary, the favorable toxicity profile of HMAs has made it feasible to treat a larger group of AML patients. However, it should be noted that the prospect of these patients is still very unfavorable with an OS of only 6-13 months.

Position of azacitidine and decitabine among conventional treatment types
Now that azacitidine has been approved for higher-risk MDS and both azacitidine and decitabine have been approved for older AML patients, the question is how to define their position among other treatment types and how further improvements can be made on this backbone. In higher-risk MDS, azacitidine is currently considered as standard of care in the large group of patients ineligible for allo-HCT, given the reported survival benefit over conventional care. Azacitidine largely replaced BSC and low-dose cytarabine in MDS patients, as improved survival, improved response rates, and lower toxicity have been observed compared to BSC and low-dose cytarabine.\textsuperscript{6,24} Further, unlike low-dose cytarabine, azacitidine has been shown to improve outcome of patients with adverse genetic risk, including the very-poor risk monosomal karyotypes.\textsuperscript{25}

For AML patients, results from this thesis and previous studies suggest that treatment with HMAs (decitabine and azacitidine) must be strongly considered instead of BSC in all patients who are able and willing to undergo treatment.\textsuperscript{7,16,18} Of the 114 BSC patients in our cohort, 54% received 6-mercaptopurine (6-MP; 200-250 mg 2 times a week). Median OS was improved with 6-MP but still unfavorable compared to azacitidine (4.8 months with 6-MP versus 1.9 months without 6-MP versus 13.3 months with azacitidine, \(p < 0.001\); Figure 1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Overall survival in patients treated with best supportive care (BSC) only, BSC plus 6-mercaptopurine (BSC + 6MP), or hypomethylating agents (HMA).}
\end{figure}

To correct for the time for starting 6-MP, which could bias a favorable outcome in the 6-MP group, a time-dependent survival analysis was conducted in STATA 13.0. All BSC (\(N = 53\)) and BSC + 6-MP (\(N = 61\)) patients started in the BSC group. At the time they started 6-MP treatment, patients were censored in the BSC group and were counted at risk in the BSC + 6-MP group.
The results of our cohort of consecutive older AML patients indicate that azacitidine and intensive chemotherapy might induce a similar survival benefit in a subgroup of patients, even though response rates were higher with intensive chemotherapy (75% remissions versus 42% with azacitidine (Chapter 5)). The comparable OS despite lower response rates is in line with the general clinical experience that achievement of CR or PR is not a prerequisite for prolonged OS by azacitidine. To address this matter, new response criteria have recently been proposed for AML patients treated with HMAs, which include HI in the absence of bone marrow blast clearance as valid response. Similar OS with HMAs and intensive chemotherapy was also observed in a large Spanish cohort of 671 older AML patients, in a subgroup analysis of the AZA-AML-001 trial, and in the AZA-001 trial. Superior OS has been reported with HMAs compared to intensive chemotherapy in patients over 70 years of age. However, also the opposite has been reported, with significantly superior survival after intensive chemotherapy compared to azacitidine in a retrospective study of 334 AML patients aged 60 years or older. Anyhow, older AML patients treated with intensive chemotherapy generally have inferior outcome compared to the young, which provides opportunities for less toxic therapies such as HMAs. Future research is needed to more precisely determine which subgroups (e.g. patients >70 years old, patients with monosomal karyotypes, etc.) benefit more from HMAs and which subgroups benefit more from intensive chemotherapy. Data from our extended patient named program and from others indicates that the outcome with azacitidine is poorer in AML patients with proliferative disease, reflected by higher WBC counts (>15 x 10^9/L), suggesting that the hypomethylating effects are slow-acting and not dependent on the rate of cell proliferation. With decitabine, however, a trend towards improved outcome has been observed regardless of baseline WBC count in a post-hoc analysis of a large phase III trial.

Since allo-HCT is currently the only potential curative therapy for MDS and AML, an interesting treatment approach to assess in older patients would be to combine cytoreductive therapy with an HMA and allo-HCT. It should be noted that most treatment schedules with HMAs (except for the 10-day decitabine schedule) are associated with lower response rates compared to IC and that especially remission without minimal residual disease is associated with favorable outcome after allo-HCT. Nevertheless, retrospective studies in MDS patients indicate that azacitidine followed by allo-HCT is equally effective as intensive chemotherapy followed by allo-HCT in indolent disease. HMAs may trigger an enhanced graft-versus-leukemia effect via increasing regulatory T-cell numbers, activating immune cells and upregulation of tumor antigens through hypomethylation. First results of the addition of decitabine to a fludarabine/total body irradiation-conditioning schedule followed by allo-HCT are encouraging. Currently, a prospective trial (EORTC-1301) randomizing older AML patients for induction therapy with decitabine versus conventional chemotherapy before allo-HCT is ongoing.

Furthermore, different schedules of HMAs may be feasible and effective. For example, a combination of intensive chemotherapy and an HMA followed by allo-HCT might be an effective treatment strategy in fit older patients, as different cell death pathways in the heterogeneous leukemia cell population will be targeted. First results indicate that combining azacitidine or
decitabine with intensive chemotherapy is feasible in older AML patients and induces favorable response rates. Further, azacitidine may be used as consolidation therapy after allo-HCT, to prevent relapse and to augment the graft-versus-leukemia effect. Encouraging results indicate that low-dose azacitidine (35-50 mg/m² for 5 days/4weeks) is feasible after allo-HCT, is associated with low incidence of graft-versus-host disease, and can be applied at a dose of 75 mg/m² for 7 days in case of minimal residual disease. If allo-HCT is not feasible due to unavailability of a matched donor or frailty, the ideal consolidation therapy in older AML patients is undetermined. Post-remission maintenance therapy with azacitidine after intensive chemotherapy seems safe and feasible in older AML patients. A randomized trial (HOVON 97) to evaluate azacitidine maintenance versus no maintenance after intensive chemotherapy is ongoing.

As both azacitidine and decitabine are currently registered for treating older (≥65 years) AML patients ineligible for intensive chemotherapy and/or allo-HCT, the question arises which drug is preferred. In this perspective it should be noted that 80% of azacitidine is incorporated into RNA and 20% in DNA, while 100% of decitabine is incorporated into DNA. Different gene expression profiles have been observed in cell lines after treatment with azacitidine and decitabine, suggesting different effects of these drugs on cell biology. In our single-center cohort, the number of AML patients treated with decitabine was too small to compare its efficacy with azacitidine. A recent meta-analysis and a retrospective analysis comparing azacitidine with decitabine in MDS patients demonstrated better results of azacitidine treatment, especially in patients older than 75 years and patients with IPSS risk ≥3. However, the decitabine dosing schedules probably were suboptimal. Inversely, a retrospective study comparing outcome after intensive chemotherapy with outcome after HMAs (both azacitidine and decitabine) revealed superiority of decitabine. Therefore, comparative studies of azacitidine and decitabine (including the 10-day intravenous 20 mg/m² dosing schedule) in MDS and AML patients will be of interest.

Prognostic and predictive factors in patients treated with hypomethylating agents

Response to HMAs requires time and is usually observed after several treatment cycles. Therefore it is recommended to administer at least six cycles of azacitidine or four cycles of decitabine (5 days schedule) before discontinuation. In view of the long time frame and costs (azacitidine costs about € 5.300 per 7-day cycle and decitabine costs about €7.000 per 5-day cycle in the Netherlands), it would be of value to have predictive markers for response. Globally used prognostic scores, including the IPSS and the IPSS-R in MDS and the cytogenetic/molecular risk stratification in AML, provide information on the likely outcome of the disease, but are not designed to predict treatment benefit. To more precisely estimate the progression-free survival and OS, specific prognostic scores in patients treated with HMAs have been designed. The prognostic score of Itzykson et al. includes WHO performance score, circulating blasts, red blood cell transfusion dependency and cytogenetics (good versus intermediate versus poor), and assigns patients up to 30% bone marrow blasts in three risk groups with significantly different OS. In the Dutch named patient program, we were able to confirm the prognostic value of this scoring system in azacitidine-treated patients. For unfit AML patients treated with azacitidine, the
European ALMA score has been developed, which uses the WHO performance score, WBC count and cytogenetics (normal versus abnormal) to discriminate between three risk groups with different OS. These prognostic scores are often used to guide decisions in favor or against azacitidine treatment, although they formally do not predict treatment benefit. Ideally, prospective or retrospective data from a randomized trial should be used to identify response predictors. In absence of these data, prognostic factors for response to treatment (instead of survival) or early markers for response may indicate treatment benefit.

Figure 2. Overall survival by platelet increase after the first month of HMA treatment
A) OS by platelet (PLT) count ratio of MDS, CMML and AML patients treated within the extended Dutch azacitidine named patient program. Patients without available information on platelet counts at start of the second cycle (N = 15) were excluded. B) OS by PLT count ratio of AML patients from our single-center cohort treated with azacitidine (N = 36) or decitabine (N = 7). Patients without available information on platelet counts at start of the second cycle (N = 15) have been excluded. Platelet ratios were determined by dividing platelet counts at start of the second azacitidine cycle by platelet counts at start of the first cycle. Patients who received platelet transfusions within 10 days before the second cycle were considered as PLT ratio ≤1.

In the Dutch azacitidine named patient program we identified a possible early marker for treatment benefit. Platelet doubling after the first cycle of azacitidine was observed in a small group of patients (about 15%) and was an independent prognostic factor for favorable OS, although statistically not significant in multivariate analysis. Importantly, also in the majority of patients who did not show early platelet increase, responses were observed. Therefore, platelet doubling could be regarded as encouragement to continue treatment, while lack of platelet doubling should not be used to withhold further azacitidine treatment. We re-assessed the extended named patient program in January 2015 (3.5 years later) to evaluate the prognostic value of early platelet increase on long-term survival. After this longer follow-up time, median OS of patients with platelet doubling after the first azacitidine cycle was 24.3 months compared to 19.3 months in patients with a less than 2 fold platelet increase, and 12.7 months in patients without platelet increase, which did not reach statistical significance (p = 0.55; Figure 2A). We also assessed early platelet increases in our cohort of older AML patients treated with azacitidine or decitabine. Only six of 43 evaluable patients showed platelet doubling after the first cycle.
Therefore, we combined this group of patients with patients who had a platelet increase less than 2 fold. AML patients with a more than 1 fold platelet increase after the first cycle (N = 21 azacitidine/1 decitabine) had a significantly longer median OS (28.2 months) compared to patients without any platelet increase (N = 15 azacitidine/6 decitabine; median OS 8.4 months; p = 0.003; Figure 2B).

A prognostic value of early platelet increase during HMA treatment has also been observed by other groups. Zeidan et al. validated platelet doubling after the first azacitidine cycle in a cohort of 102 patients with MDS or AML with less than 30% bone marrow blasts and confirmed a longer median OS in patients with platelet count doubling (21.0 months) compared to those without (16.7 months, HR 1.88 (95% CI 1.03-3.40), p = 0.04). Raffoux et al. reported that early platelet responses were associated with higher remission rates in AML and MDS patients treated with azacitidine, valproic acid, and all-trans retinoic acid. In the Australian azacitidine named patient program, a non-significant trend towards longer OS was observed in AML patients with platelet count doubling after one cycle (N=23) compared to others (N = 220; median OS 574 versus 330 days, p = 0.141). Van den Bosch et al. and Jung et al. observed superior OS in MDS patients treated with decitabine who had an early platelet response. Apparently HMAs affect megakaryocyte development. A mouse study suggests that decitabine enhances megakaryocyte maturation and platelet release. It is unclear whether the aberrant MDS or AML cells or the residual normal cells are responsible for the increased production. Several studies suggest that normal hematopoietic cells can be stimulated to megakaryocyte differentiation. Experimental treatment with decitabine in eight patients with sickle cell anemia led to an increase in platelet counts and bone marrow megakaryocytes in all patients. Also in five patients with β-thalassemia and fifteen patients with metastatic lung cancer, experimental treatment with decitabine resulted in 1.3 to 3 fold increases in platelet counts. These data are in line with the observation that cytogenetically normal CD34+ cells in MDS patients expand in response to treatment with erythropoietin and granulocyte-colony stimulating factor. On the other hand, increases of dysplastic megakaryocytes have been observed in at least one study in MDS patients who had a platelet response upon treatment with decitabine, suggesting that also dysplastic cells can be stimulated to production of platelets.

### Gene mutations and response to HMAs

In recent years, various gene mutations have been identified in MDS and AML by next-generation sequencing. Increasing evidence demonstrates the association of these mutations with clinical outcome (Table 2). Some mutations, such as TET2 and DNMT3A mutations, are generally associated with adverse outcome, but are predictive for improved response to HMAs. One of the most unfavorable mutations is mutation of TP53. Very poor survival has been reported with conventional therapy. Although we also observed poor survival in our cohort of AML patients, our results indicate that patients with TP53 mutations may benefit from hypomethylating agents with similar response rates compared to TP53-wild type patients and temporary suppression of
the TP53-mutated AML clone. However, prospective studies are needed to determine whether TP53-mutated patients indeed benefit from HMAs.

Table 2. Mutations and their clinical significance in MDS and AML

<table>
<thead>
<tr>
<th>Category</th>
<th>Mutant gene</th>
<th>Frequency in MDS (%)</th>
<th>Frequency in AML (%)</th>
<th>Clinical findings and prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA methylation</td>
<td>DNMT3A</td>
<td>10-15</td>
<td>20-50</td>
<td>Adverse; but favorable response to HMAs</td>
</tr>
<tr>
<td>DNA demethylation</td>
<td>TET2</td>
<td>20-30</td>
<td>10-20</td>
<td>Poorer in int-risk AML; but favorable response to HMAs</td>
</tr>
<tr>
<td>IDH1</td>
<td></td>
<td>3</td>
<td>7</td>
<td>Poorer in FLT3-ITD-negative AML</td>
</tr>
<tr>
<td>IDH2-R140</td>
<td></td>
<td>5</td>
<td>2</td>
<td>Adverse</td>
</tr>
<tr>
<td>IDH2-R172</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT1</td>
<td>&lt;1</td>
<td>9</td>
<td></td>
<td>Poorer in NK-AML</td>
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<tr>
<td>Activated signalling</td>
<td>FLT3-ITD</td>
<td>&lt;1</td>
<td>27</td>
<td>Poorer in int-risk AML</td>
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<td>FLT3-TKD</td>
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<td>Variable according to study</td>
<td></td>
</tr>
<tr>
<td>KIT</td>
<td>&lt;1</td>
<td>4</td>
<td></td>
<td>Poorer outcome in CBF AML</td>
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<tr>
<td>Myeloid transcription factors</td>
<td>RUNX1</td>
<td>10</td>
<td>5</td>
<td>Adverse</td>
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<tr>
<td>bi-CEBPA</td>
<td></td>
<td>&lt;1</td>
<td>4</td>
<td>Favorable</td>
</tr>
<tr>
<td>Tumor suppressor/multifactorial</td>
<td>TP53</td>
<td>5-18</td>
<td>8-16</td>
<td>Adverse</td>
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<tr>
<td>NPM1</td>
<td>&lt;1</td>
<td>33</td>
<td></td>
<td>Favorable in absence of FLT3-ITD and mutant DNMT3a</td>
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<tr>
<td>Chromatin regulation</td>
<td>ASXL1</td>
<td>15-25</td>
<td>5</td>
<td>Poorer in int-risk AML</td>
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<td>MLL-PTD</td>
<td>&lt;1</td>
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<td></td>
<td>Adverse</td>
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<td>Splicosome</td>
<td>SF3B1</td>
<td>25-30</td>
<td>3</td>
<td>Favorable in MDS</td>
</tr>
<tr>
<td>SRSF2</td>
<td>15</td>
<td>2</td>
<td></td>
<td>Adverse in MDS</td>
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*ITD, internal tandem duplication; TKD, tyrosine kinase domain; int, intermediate; HMAs, hypomethylating agents; bi-CEBPA, bi-allelic CEBPA mutations. Table based on Grimwade et al.252 and 253-256.*

**Proteasome inhibition in AML**

To improve cure rates in AML patients, investigation of new therapeutic strategies based on differences between AML (stem) cells and normal hematopoietic stem- and progenitor cells is essential. One of the potential treatment targets is the transcription factor NF-κB, which is constitutively activated in the leukemic cells of a majority of AML patients and appears to be important for the survival of AML blasts.81,82 Various pathways have been proposed to be involved in constitutive NF-κB activity, including genetic aberrations leading to increased NF-κB activation (e.g. AML1-ETO translocation, C/EBPa mutation, deletion of 5q), autocrine/paracrine signaling loops, upregulation of upstream components of the NF-κB pathway such as IRAK1, TAK1, and BTK; and increased proteasome activity leading to increased degradation of the NF-κB-inhibitory protein IκBα.81 Based on these pathways, various treatment options have been proposed to inhibit NF-κB. Inhibition of BTK by ibrutinib for example resulted in cell death and decreased NF-κB
activity in primary AML cells, and showed cytotoxic effects in chronic lymphocytic leukemia in phase I and II clinical studies. IRAK1 is recently shown to be inhibited by the FLT3/JAK inhibitor pacritinib in AML cells.

In this thesis, we evaluated the effects of the proteasome inhibitors carfilzomib, oprozomib and bortezomib on patient-derived AML cells in vitro. We observed a cytotoxic effect of carfilzomib on both primitive AML CD34+ cell fractions and more mature AML CD34- cell fractions, whereas the first-generation proteasome inhibitor bortezomib mainly targeted more mature AML CD34- cell populations. To evaluate whether carfilzomib induces apoptosis in AML CD34+ cells with acceptable effects on normal tissue, in vivo studies are required. Oprozomib, when tested at equimolar concentrations, showed limited effectiveness. However, also the effects on normal CD34- cells were smaller, suggesting that oprozomib could be safely added at higher concentrations that may result in higher effectiveness. Future experiments are needed to define the optimal dose schedule for oprozomib to target primitive AML cells. Furthermore, we observed that the anti-apoptotic protein MCL-1 was upregulated in AML cells incubated with carfilzomib, oprozomib, or bortezomib. Inhibition of MCL-1 by obatoclax enhanced the apoptotic effects of the proteasome inhibitors, suggesting that combinations of proteasome inhibitors with drugs that target anti-apoptosis pathways may be worthwhile to further investigate. Currently, several drugs that target MCL-1 or other anti- or pro-apoptotic BCL-2 family members are under investigation, including the pan-BCL-2 inhibitors obatoclax and gossypol, the MCL-1-specific inhibitors maritoclax and MIM1, and the Bcl-X/L/Bcl-2/Bcl-w inhibitors ABT-737 and ABT-263. All of these compounds induced apoptosis in various cancer cells in vitro and in vivo, especially in hematologic malignancies. However, phase I and II clinical studies with some of these drugs as single agents have reported only modest effects so far. Still, given the preclinical results, studies with drug combinations with BCL-2 family inhibitors may be of interest.

In light of predicting response to treatment, we assessed baseline chymotrypsin-like activity in several sensitive and less-sensitive AML samples. In this limited amount of samples, we did not observe an association of chymotrypsin-like activity with sensitivity to carfilzomib. This might be related to variable affinity of carfilzomib to chymotrypsin-like activity sites of the constitutive proteasome versus the immunoproteasome present in hematopoietic cells. A recent study suggests that higher ratios of immunoproteasome versus constitutive proteasome subunit expression are associated with sensitivity of AML cells to carfilzomib. Future studies to assess possible associations between response and chymotrypsin-like activity, as well as other related proteins such as NF-κB and Nrf2, are of interest and could shed more light on the mechanism of action of carfilzomib in AML cells.

In AML patients, carfilzomib has not been tested yet, but bortezomib treatment has been associated with some favorable first results. In a phase II clinical trial in AML patients aged 60 to 75 years, efficacy and safety of adding bortezomib to cytarabine/daunorubicine induction and intermediate-dose cytarabine consolidation therapy was assessed. Results revealed favorable CR rates of 65% with a median disease-free survival of 8 months and median OS of 12 months. Further, it was concluded that 1.3 mg/m² bortezomib combined with intensive chemotherapy had
an acceptable toxicity profile, although 11 out of 95 patients developed grade 3 sensory neuropathy. Since carfilzomib has not been associated with neurotoxicity or other severe side-effects, carfilzomib is a promising alternative to bortezomib. Several subgroups of patients may be more sensitive to proteasome inhibition. For example, AML cell samples bearing a FLT3-ITD mutation are shown to be more sensitive to bortezomib compared to wild type cells, which was related to bortezomib-induced degradation of FLT3 and FLT3-ITD by autophagy. In the above mentioned phase II study, six out of eight (75%) patients with FLT3-ITD achieved CR.

Interestingly, a DNA hypomethylating effect of bortezomib has been observed in AML cells, which was related to downregulation of DNMT1 via interfering with its Sp1/NF-κB transcription complex. Therefore, addition of a proteasome inhibitor may enhance the efficacy of HMAs, which inhibit DNMTs by direct interaction. A phase I clinical trial combining bortezomib with decitabine in poor-risk AML patients reported CR in 9/17 patients with good initial tolerability but occurrence of neurotoxicity after two cycles requiring discontinuation of bortezomib in three patients. Unfortunately, a subsequent randomized study of decitabine versus decitabine plus bortezomib was closed prematurely because an interim analysis indicated that the combination was unlikely to be superior to decitabine alone. Another phase I trial combined bortezomib with azacitidine in patients with relapsed or refractory AML. Of these 23 poor-risk patients, five achieved a remission after a median of (only) 2 cycles (range 1-12+). However, again significant neurotoxicity was reported, urging the need of replacing bortezomib in future studies by proteasome inhibitors with lower toxicity profiles, such as carfilzomib.

Other new treatment strategies in higher-risk MDS and AML

Currently, various other promising therapeutic approaches that may improve future outcome in higher-risk MDS and AML patients are being investigated. The novel hypomethylating agent guadecitabine is a dinucleotide of decitabine and deoxyguanosine that is resistant to degradation by cytidine deaminase, resulting in a prolonged half-life compared to azacitidine and decitabine, which have a half-life of less than 30 minutes. The prolonged half-life could potentially improve response rates especially in lower proliferative disease, since azanucleosides must be incorporated into DNA during the S-phase of cell division to exert a hypomethylating effect. Phase I and II studies in AML patients ineligible for intensive chemotherapy demonstrated that subcutaneous guadecitabine at 60 mg/m² for 5 days is well tolerated and is clinically active with CR rates of 57% and median OS of 10.5 months. A phase III study is currently being conducted.

Another strategy to improve treatment results with HMAs is addition of histone deacetylase (HDAC) inhibitors, since in vitro studies revealed a synergistic effect of histone deacetylation and DNA hypomethylation on re-expression of genes silenced by malignant transformation. However, clinical studies investigating combinations of an HMA with the HDAC inhibitor valproic acid or entinostat not only showed lack of improved response and survival, but also found lower degrees of hypomethylation when both drug types were combined, suggesting pharmacodynamic antagonism. Nevertheless, combination of the HDAC inhibitor pracinostat with azacitidine
induced promising remission rates of 54% with an estimated 1-year survival rate of 60% in AML patients aged ≥65 years ineligible for intensive therapy. Further, several low-intensity drugs and drug combinations have recently reached phase II and III clinical trials, however with at the moment only modest efficacy in older AML and higher-risk MDS patients. Gemtuzumab ozogamicin is an anti-CD33 antibody conjugate that in combination with low-dose cytarabine induced higher CR rates compared to single agent low-dose cytarabine (30% versus 17%, \( p = 0.006 \)), but did not improve OS.\textsuperscript{100} Likewise, combination of gemtuzumab ozogamicin with azacitidine or decitabine led to increased OS rates but not to improved OS rates in phase II studies compared to historical data, except for poor risk AML patients who seemed to benefit from azacitidine plus gemtuzumab ozogamicin.\textsuperscript{101,102} Volasertib is a selective cell cycle kinase inhibitor that targets Polo-like kinase 1 inducing cell cycle arrest and apoptosis. Combination with low-dose cytarabine resulted in improved remission rates (31.0% versus 13.3%, \( p =0.052 \)) and median OS rates (8.0 versus 5.2 months, \( p = 0.047 \)), but also increased neutropenic fever/infections and gastro-intestinal effects compared to low-dose cytarabine alone in AML patients ineligible for intensive therapy.\textsuperscript{103} Interestingly, responses were observed across all genetic risk groups, including 5/14 patients with adverse risk. A phase III study of volasertib plus low-dose cytarabine and studies combining volasertib with decitabine or intensive chemotherapy are currently being conducted. Tipifarnib, a farnesyltransferase inhibitor affecting Ras-signaling, might improve survival as maintenance therapy in AML patients at high risk for relapse.\textsuperscript{104} The Aurora B kinase inhibitor barasertib and the nucleoside analogue prodrug sapacitabine did not appear to improve outcome in AML.\textsuperscript{105}

An interesting upcoming development is the emergence of personalized therapy or ‘precision medicine’ in AML, which takes into account inter-patient differences in disease- and patient characteristics and possible intra-patient differences due to clonal evolution over time.\textsuperscript{106,107} Besides currently available therapies, emerging targeted therapies such as inhibitors of FLT3, isocitrate dehydrogenase 1 (IDH1), IDH2, NF-κB, or bromodomain and extra terminal protein (BET), will in particular fit well in this concept.

**Age-related changes in hematopoiesis and AML**

To improve outcome in older AML patients, it is important to enlarge our insight in the causes of their poor outcome. It has become increasingly clear that differences exist between young and old individuals in normal hematopoiesis. Ageing has been associated with an increased prevalence of clonal hematopoiesis, considered as a pre-leukemic state.\textsuperscript{108} Further, whereas normal hematopoiesis under physiological conditions is largely maintained by short-term hematopoietic stem cells (HSCs) and progenitors, increased numbers and increased activity of HSCs is observed with ageing.\textsuperscript{109,110} These aged HSCs seem to be skewed to the myeloid compartment and have a diminished function as reflected by a reduced self-renewal capacity and reduced engraftment following stem cell transplantation.\textsuperscript{109,111,112} Besides contributing to a higher risk of developing myeloid malignancies, age-related changes might also limit peripheral blood recovery after
treatment, for example due to possibly increased vulnerability of normal HSCs to anti-leukemic therapy.

In AML, older age is associated with increased numbers of gene mutations in intermediate- and adverse-risk AML.\textsuperscript{113,114} Also the type of mutations is different in older patients, as mutations more frequently occur in \textit{ASXL1, MLL, RUNX1, and TET2}, while \textit{NRAS} mutations appear less often in older AML patients.\textsuperscript{114} These data suggest that older AML patients often have a disease with different properties than younger AML patients, which might confer different sensitivity towards treatment. More research is needed to further characterize AML cells of older patients and to adapt treatment to their specific alterations.

Further, age-related changes may contribute to the higher relapse rates in older AML patients after treatment. As preleukemic cells are shown to be able to survive therapy and to generate new clones or activate dormant clones that initiate relapse\textsuperscript{115,116}, the higher prevalence of (preleukemic) clonal hematopoiesis in older individuals contains an increased risk of relapse. The appearance of TP53-overexpressing cells upon relapse that we observed in one of our azacitidine-treated AML patients in Chapter 5 might have been due to the outgrowth of a small (preleukemic) clone. In addition, due to impaired sensitivity of older AML cells, low numbers of AML cells might persist during remission, causing relapse. In Chapter 5, two patients showed re-appearance or expansion of TP53-overexpressing cells at time of relapse, suggesting persistence of small amounts of TP53-mutated AML cells during HMA treatment in remission.

CONCLUSIONS

In conclusion, in this thesis we have shown that azacitidine is effective and feasible in daily clinical practice in higher-risk MDS patients and older AML patients, including AML patients with more than 30% bone marrow blasts or with \textit{TP53} mutations. We demonstrated that azacitidine in older AML patients is associated with better outcome compared to BSC, and with similar outcome compared to intensive chemotherapy not followed by allo-HCT. Further, we showed that the second-generation proteasome inhibitor carfilzomib reduces survival of primitive AML cell fractions \textit{in vitro}. More research is required to improve current treatment with HMAs, to guide treatment decisions in higher-risk MDS and older AML patients, and to assess \textit{in vivo} and clinical effectivity of new possible treatment strategies including carfilzomib.
REFERENCES


Summary, discussion and future perspectives


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