Molecular mechanisms of bortezomib resistance in acute leukemia

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2017

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Chapter 1

General introduction

INTRODUCTION

Of all pediatric malignancies, acute leukemia is the most common type. Acute pediatric leukemia can be divided in two major subgroups; Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML). ALL represents the major part (85%) with a yearly incidence of 30 cases per million children in the Netherlands, while AML has an incidence around 6,5 per million and CML 1 per million.[1]

Yearly incidence of AML in adults according to data from the USA is increasing with age starting at an incidence of 9.6 per million between the age of 20 to 39 years, to an incidence of 32 per million above the age of 75 years. The opposite is seen in adult ALL: the incidence of 14.4 per million between the age of 20 to 39 years to an incidence 4.7 per million above the age of 75 years.[2] This thesis will focus on the more frequent forms of acute leukemia, namely ALL and AML.

With a 5 year overall survival (OS) 83-94%[3,4] , the prognosis of pediatric ALL is considerably better as compared to adults (OS 15-35%, dependent on the age of the studied adult population).[2,5] Similar difference in prognosis between children and adults is seen AML with a 5 years OSS of respectively 65%-70%[6-8] and 10-45% (dependant on the age of the studied adult population).[9] Therefore, new treatment options are needed to improve the outcome of these hematological malignancies. In this thesis, we investigated a novel class of drugs in the treatment of acute leukemia, i.e proteasome inhibitors (PI) with Bortezomib (BTZ) as its prototypical representative. In our studies we particularly focused on factors that contribute to sensitivity and resistance of acute leukemias to BTZ.

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

ALL is caused by genetic alterations in lymphoid progenitor cells resulting in proliferation and clonal expansion.[10] Importance of genetic background has been emphasized in children with Down syndrome who have a 25-fold increased chance of developing ALL as compared to the general population.[11] In addition, recently inherited germline mutation in the PAX5 gene has been shown to induce susceptibility to pre-B cell ALL in rare cases of familial hereditary ALL.[12] The role of the pre-leukemic TEL–AML1 fusion gene is less clear. While this genetic aberration is present in a leukocyte subpopulation in around one percent of healthy newborn infants, only a small percentage of these children will develop ALL in later life.[13] In addition, non genetic factors have been described to increase the chance of developing ALL. Ionizing radiation has been shown to be a causal factor in the development of childhood ALL.[14,15] Moreover, the timing and severity of infections during childhood have been associated with risk of developing

ALL (reviewed by Greaves *et el.*[16]). In particular delayed exposure to infections has been correlated to increased ALL risk[17,18] and an early exposure to infections through e.g. daycare, reduces this risk.[19,20]

Prognostic factors

Over the last decades, the survival prognosis of pediatric ALL has increased dramatically from a 5 year overall survival of 21% in 1960 to 83-94% in the more recent treatment protocols.[3,4,21] However, still 20% to 25% of patients relapse during or after completion of current protocols for newly diagnosed pediatric ALL and need to undergo additional therapy.[4] Patients between 18 and 60 years though have a considerably worse prognosis with an average survival of 35%. With an average survival between 15 and 20%, patients above the age of 60 years have an even worse prognosis.[2,5] Prognosis of the individual patient is dependent on the leukemia subtype. ALL can be subdivided based on phenotype and genetic aberrations. Phenotypically, ALL can be categorized into (precursor) T-cell, precursor B-cell and mature B-cell (Burkitt). Further classification can be made based on genetic aberrations that are found in almost all of cases with pediatric ALL (Figure 1A). The most common alterations are hyperdiploidy and the translocation t(12;21) resulting in ETV6-RUNX1 (also known as TEL-AML1), which account for around 50% of the cases. The influence of the individual aberrations on prognosis has been reviewed by Pui et al. and Bjohwani et al. [22,23] Adolescents and adults, who have a worse prognosis as compared to younger children, feature a lower incidence of the prognostic favorable subtypes (e.g. ETV6-RUNX1 and hyperdiploidy) and a higher incidence of unfavorable genetic aberrations like BCR-ABL and amplification of chromosome 21 (reviewed by Schafer *et al.*[24]).

Some specific subgroups concern patients with Down syndrome, infants and children with BCR-ABL positive, and a more recently recognized class, BCR-ABL1-like ALL. Children with Down syndrome have a lower survival rate compared to the general pediatric population[25,26], probably due to a lower incidence of prognostically favorable cytogenetic risk groups in combination with a higher frequency of treatment-related toxicity (reviewed by Maloney *et al.*[27]).

While ALL in infants is relatively rare, they have a worse prognosis as compared to older children. This difference in outcome is caused by a combination of high frequency of aberrations in chromosome 11q23 region resulting in alterations of the mixed lineage leukemia (MLL) gene, high incidence of the very immature B-cell phenotype without CD10 expression and relative high white blood cell counts at diagnosis.[28,29] In this group, an adapted hybrid protocol containing elements of both ALL and AML treatment protocols has been shown to be superior over the standard ALL protocol.[30]

Translocation of chromosome 9 and 22 (Philadelphia chromosome) forms a fusion gene of BCR and c-ABL resulting in the fusion protein BCR-ABL. ALL positive for this

translocation, also have a worse prognosis when using conventional therapy. Therefore, this subgroup is currently treated with the BCR-ABL inhibitor imatinib in addition to the conventional therapy[31], which has dramatically improved outcome in this subgroup.[32] In addition, gene expression profiling identified a poor prognostic group harboring a BCR-ABL like gene expression profile, without the targetable fusion protein.[33,34]

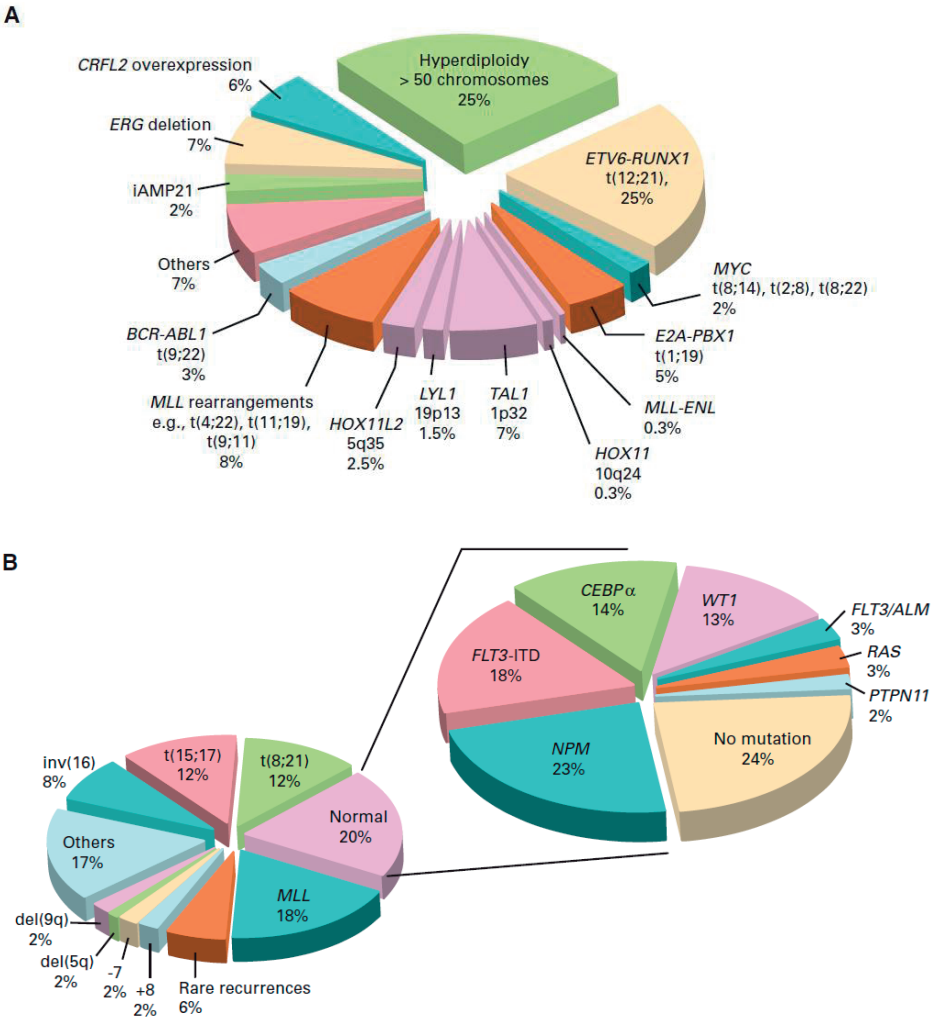

Figure 1. Estimated frequency of specific genotypes in childhood leukemias.
(A) Genetic abnormalities in acute lymphoblastic leukemia (ALL). Genetic lesions exclusively seen in T-cell ALL are indicated in purple. **(B)** Genetic abnormalities in acute myeloid leukemia (AML). Panel to the left demonstrates the most common karyotypic alterations. Mutation profile in patients with normal karyotype is shown in the right panel (reprinted with permission of Pui et al. [2] and Reaman and Smith).

Treatment

Leukemias are treated with combinations of chemotherapy of which each drug harbors a different mechanism of action. An overview of cytotoxicity mechanisms of the most frequently used chemotherapeutics in leukemia treatment is depicted in Figure 2.

Most treatment protocols of ALL consist of an induction, consolidation, CNS-directed, re-induction and maintenance phase.[35] The induction therapy usually consists at least of a glucocorticoid, vincristine, anthracyclines and L-asparaginase. This first phase is designed to induce cytomorphological remission. In the second phase, complete remission (CR) is consolidated by agents such as cytarabine (Ara-C) and cyclophosphamide. In de CNS-directed phase, high-dose methotrexate (MTX) and 6-mercaptopurine (6-MP) are administered to reduce the residual tumor load and to prevent future relapses in the central nervous system. In both the induction and consolidation phase, the systemic therapy is combined with intrathecal chemotherapy for the same reason. The re-induction phase resembles the induction phases and aims to reduce the relapse risk. The maintenance therapy typically has a backbone of 6-MP and MTX, in some protocols supplemented with vincristine and prednisone or dexamethasone pulses (reviewed by Pieters *et al.* [36])

Commonly, patients are stratified in risk groups. In the current Dutch Childhood Oncology Group (DCOG) ALL protocol (ALL-11), patients are stratified in standard risk (SR), medium risk (MR) and high risk (HR) groups, based on central nervous system (CNS) involvement or testis involvement at diagnosis, prednisone response at day 8, cytomorphological remission status at day 33, minimal residual disease (MRD) at day 33 and 79 and presence of t(4;11)(q11;q23) translocation or the corresponding fusion. Furthermore, the MR group is further subdivided based on TEL/AML1 fusion gene, Ikaros (IKZF1) gene status and Down syndrome comorbidity. Based on this risk stratification, the consolidation, re-induction and maintenance phases are adapted. In the group without IKZF1 and presence of Down syndrome or TEL/AML1, anthracyclines are omitted. In case of IKZF1 presence, the maintenance therapy is prolonged with 1 additional year.[37] Due to the good overall survival of the chemotherapy-based protocols in children, allogeneic stem cell transplantation in this group is only reserved for the very-high-risk patients.[38]

Historically, patients treated in adult ALL trials have a considerably worse prognosis as compared to their pediatric ALL counterpart.[2,5] This difference could only partly be explained by genetic differences.[39] Since in many countries adolescents were referred to either dedicated pediatric or adult hematology departments for the treatment of their ALL, retrospective studies could be performed to compare both treatment strategies.[40,41] Notably, the pediatric protocol turned out to be superior, at least partly explainable by more intensive regimen. This led to the introduction of pediatric treatment based protocols in older adolescents as well as young adults.[4,42]

In the Netherlands, adult ALL patients are currently treated according to the HOVON 100 ALL / EORTC 06083 study.[43] In this protocol, chemotherapy is largely comparable with the pediatric protocol, although the intensity is reduced for patients above the age of 40 years. Since the prognosis of adult ALL is considerably worse, adult patients in the standard risk and high risk groups are eligible for allogeneic Stem Cell Transplantation (allo-SCT), although it is a matter of debate whether this improves outcome in general in ALL. Another fundamental difference is the use of irradiation. While cranial irradiation is still incorporated in adult protocols in case of involvement of (even limited) central nervous system, this step is omitted from the contemporary pediatric protocols because of severe late neurotoxicity. In addition, total body irradiation in the transplantation setting is also omitted from pediatric protocols for the same reason.[44]

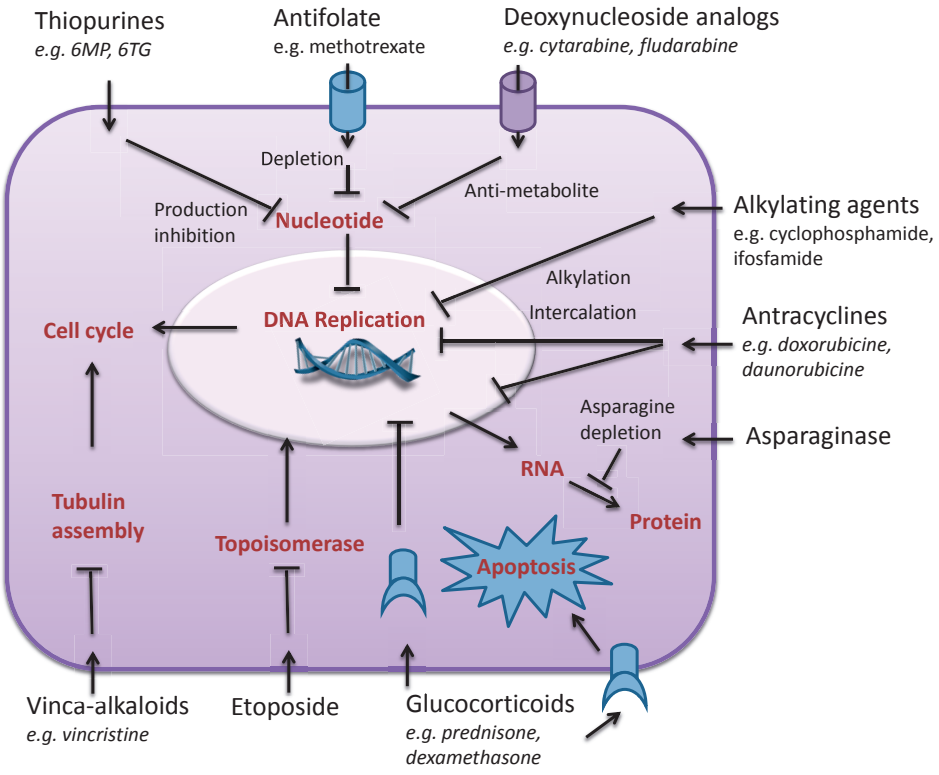

Figure 2. Overview of cytotoxicity mechanism of chemotherapy drugs commonly used in acute leukemia treatment.

ACUTE MYELOID LEUKEMIA (AML)

AML is caused by at least two different mutation types in myeloid progenitor cells; mutations that result in proliferative and survival signals, and mutations that lead to differentiation arrest or enhanced self-renewal (reviewed by Pui *et el.*[22]). Like normal hematopoietic progenitors, AML cells are hierarchically organized into compartments that contain leukemic stem cells (LSCs) that have self-renewal capacity and give rise to leukemic blast cells (reviewed by Nguyen *et al.*[45]). Etiology of AML is usually unknown although it is more often observed after chemotherapy and/or radiotherapy for a different malignancy, and several bone marrow failure syndromes including Fanconi anemia, Diamond-Blackfan anemia, Bloom's syndrome and ataxia telangiectasia. In addition, the risk of developing AML is 400-fold increased in children with Down syndrome as compared to the general population.[46]

Prognostic factors

Like in ALL, the prognosis of pediatric AML has increased substantially from a 5 year survival of 20-30% in the 1970s to 65%-70% nowadays.[6-8,47] Relapse after first remission occurs in 30-40%.[6,48] Although survival of relapsed patients is considerably worse, they still face a long-term survival of about 35% after relapse therapy.[49,50] Consistent with ALL, adults suffering from AML also experience a worse prognosis with a 5 year survival of 40-45% in adults up to 65 years, and only 10-15% above 65 years (reviewed by Ferrara and Schiffer[9]).

The subtype of AML determines actual prognosis for the individual patient. Traditionally, AML is subdivided based on the mainly morphological French-American-British (FAB) classification which consists of the subclasses M0 to M7. Due to upcoming genetic diagnostics, the significance of this classification system has decreased. An exception to this is the M3 Acute Promyelocytic Leukemia (APL) which still represents a different entity within AML with a tailored protocol establishing a better overall survival.[51] The prognosis of the majority of the AML patients is dependent on the genetic abnormalities found in their leukemic cells. For instance FMS-related tyrosine kinase 3 internal tandem duplications (FLT3-ITD) conveys a worse outcome while the fusion proteins RUNX1-RUNX1T1 and CBFB-MYH11 both have favorable prognosis. An overview of all aberrations with corresponding impact on survival is presented in recent reviews for childhood AML at diagnose[6,7,47], and relapse[52], and by Ferrara and Schiffer[9] for adults with AML. Figure 1B summarizes these genetic aberrations found in AML.

Patients with Down syndrome also are at relatively high risk to develop AML. In contrast to ALL, Down patients with AML have a better prognosis as compared to other AML patients, particularly if it concerns the entity of myeloid leukemia of Down syndrome with GATA1-mutated AML cells.[53] Given this feature, and the fact that Down patients

have a higher susceptibility to suffer from treatment related toxicities, they are treated with a reduced intensity protocol.[54-56]

As mentioned above, APL has a favorable prognosis. This subgroup usually harbors a t(15;17) translocation resulting in the PML-RARα fusion protein which renders them sensitive for the vitamin A analogue all trans retinoic acid (ATRA). This compound induces differentiation of the APL blasts which results, often in combination with additional chemotherapy, in a high treatment response rate.[57] More recently, arsenic trioxide is introduced in the treatment of APL as well, with seemingly superior results when combined with all-trans retinoic acid (ATRA).[58]

Treatment

The initial treatment of pediatric AML varies per country, but most of the treatment protocols include at least an antracycline (e.g. daunorubicin, doxorubicin, idarubicin or mitoxantrone), a topomerase II inhibitor (e.g. etoposide) and a nucleoside analogue (e.g. cytarabine). Dose and timing of the different agents varies per protocol.[7,59] Pediatric patients in the Netherlands are currently treated according to the NOPHO-DBH AML 2012 protocol. The treatment is divided in an induction phase, and a consolidation phase in some cases followed by allogeneic stem cell transplantation. In the first course of the induction phase of this protocol, patients are randomized between mitoxantrone together with cytarabine and etoposide vs. liposomal daunorubicin together with cytarabine and etoposide. The second course has a second randomization between etoposide together with Liposomal daunorubicin and cytarabine vs fludarabine together with liposomal daunorubicin and high-dose cytarabine. Standard risk patients receive 3 blocks of chemotherapy in the consolidation phase: first high-dose cytarabine with mitoxantrone, secondly high-dose cytarabine with etoposide followed by a final block containing fludarabine and cytarabine. For patients within the standard risk group with the favorable inv(16), the first consolidation block is omitted. High risk patients start with standard risk consolidation treatment and if a donor is available are transplanted after the first consolidation treatment block.[60]

Between pediatric oncology groups, patient selection for and timing of allogeneic stem cell transplantation (allo-SCT) remains a point of debate. There is consensus regarding not performing allo-SCT for low risk groups, whereas the efficacy of allo-SCT in standard risk and high risk groups varies between clinical studies, thus imposing different transplantation criteria in various treatment protocols (reviewed by Niewerth *et al.*[61]).

Treatment protocols of adult AML consist of an induction phase containing an anthracycline (usually daunorubicin or idarubicin) together with cytarabine, a consolidation phase containing high or intermediate dose of cytarabine generally followed by allo- or auto-SCT if eligible.[9] Due to higher frequency of pre-existing co-morbidities in espe-

cially older adults, this high intensity treatment protocol is not feasible in a large group of patients, thus warranting an adapted treatment protocol.[62,63] In the Netherlands, the addition of the thalidomide analogue lenalidomide to the standard remission-induction chemotherapy followed by allo-SCT is planned to be investigated in the upcoming HOVON 132 trial in adults up to age of 65.[64] For patients who are not eligible for allo-SCT, the effect of lenalidomide maintenance therapy after auto-SCT or post-induction chemotherapy is currently being investigated. The addition of maintenance therapy with the cytosine nucleoside analogue azacytidine directly following standard remission induction therapy is investigated in patients older than 60 years.[65]

LONG TERM SIDE-EFFECTS

With an improving overall survival, the importance of dealing with long-term side effects of chemotherapy and treatment in general has received considerable and increasing attention. Prolonged corticosteroid exposure is known to induce osteonecrosis[66] and bone mineral deficits (reviewed by Wasilewski-Masker[67]). Cardiomyopathy, which can cause heart failure in the long term, is a dose-limiting complication of anthracyclines.[68,69] Vinca alkaloids (particularly vincristine) are associated with the development of peripheral neuropathy during therapy, and can also induce abnormal nerve conduction that persists after the completion of therapy.[70] Lastly, classic alkylating agents like cyclophosphamide decrease fertility in a dose-dependent manner in both male and female.[71] In addition, this class of drugs as well as etoposide and anthracyclines increase the risk of developing second malignant and non-malignant neoplasms (SMN), predominantly AML or myelodysplastic syndrome (MDS).[72-76]

Cranial radiation therapy (CRT) is notorious for its long term side effects. CRT has been shown to be a important risk factor for the development of neurocognitive[77,78] and neuroendocrine[79] morbidity, stroke[80], overweight[81] and SMN especially brain tumors[73,82]. Therefore CRT is no longer used prophylactically, but reserved for CNS relapse.

Allo-SCT has also been linked to late toxicities including endocrinologic and neurocognitive abnormalities, infertility, cataract, and cardiovascular disease. In addition risk on developing SMN is increased by Allo-SCT.[83-86] Since SCT conditioning regimens contain several cytostatic drugs, part of the long-term effected including increased risk on SMN might be explained by this conditioning regime.[72] Moreover, total body irradiation (TBI), used in combination with chemotherapy in preparation for Allo-SCT, shows besides the side effect of CRT, dysfunction of several other organs within the irradiated fields including gonads[87,88], bones[89], kidneys[90], lungs and eyes[91]. Furthermore, Allo-SCT can be complicated by chronic Graft Versus Host Disease (cGvHD) which occurs in around 15–25% of the children undergoing Allo-SCT.[92] It potentially affects several

organ systems, most commonly the skin, eyes, oral cavity, gastrointestinal tract, liver and lungs.[93] Although the long-term prognosis of cGvHD for children is somewhat better than for adults, treatment complications including severe infections, are still an important cause of death.[94]

Besides agent-specific toxicity, several long-term effects have been noted after various combination treatment protocols, including growth retardation[95], increased incidence of stroke[80] and neuropsychological sequelae consisting of lower IQ, attention, visual-perception, mathematics and reading achievement, and verbal memory than controls.[96]

DRUG RESISTANCE

Despite an increasing efficacy of current conventional chemotherapeutic regimens, resistance to treatment remains a major challenge to deal with in clinical practice. Although cellular drug resistance often involves a complex multi-factorial process, it can be dissected in several underlying mechanisms, including reduced intracellular drug availability due to defective uptake or enhanced extrusion, impaired drug activation, drug catabolism or sequestration, alterations of the target pathways, and changes in the activation of the apoptotic machinery.[39,97-99] A summary of the different drug resistance mechanism is depicted in Figure 3.

Drug efflux can be facilitated through upregulation of ATP-binding Cassette family transporters, e.g. ABCB1 (P-Glycoprotein, MDR1), ABCC1 (MDR-associated protein, MRP1) and ABCG2 (Breast Cancer Resistance Protein, BCRP). Each member of this family of proteins is capable of extruding a variety of antileukemic drugs from cells and thereby preventing cytotoxic effects (reviewed in [100-102]). In addition, reduced drug uptake by down regulation of the human equilibrative nucleoside transporter 1 (hENT) can confer cytarabine resistance in pediatric AML.[103]

Alterations in the targeted pathway itself represent another mechanism of drug resistance. For example, deletions in the glucocorticoid receptor (GR) gene or one of the genes involved in the GR pathway may contribute to glucocorticoid resistance in ETV/RUNX1 positive relapsed ALL.[104] In the context of MTX, both mutations in the reduced folate carrier (RFC), the dominant MTX uptake transporter, as well as aberrant splicing of the enzyme folylpolyglutamate synthetase (FPGS), leading to defective MTX polyglutamylation, are common causes of anti-folate resistance.[105-109] Beyond this, polymorphic and expression variations in folate/MTX pathway genes can also contribute to inter-individual differences in response to this antifolate drug.[110-112]

An important signal transduction pathway attenuating apoptosis-induced cell death is the Nuclear Factor kappa B (NF-κB) pro-survival pathway. This pathway is upregulated in pediatric ALL[113,114] as well as in AML[115]. It is associated with high white blood cell count

(WBC) at diagnosis[116] and exerts several anti-apototic effects (reviewed by Hoesel *et al.*[117]). Furthermore, the NF-κB complex is able to inhibit glucocorticoid effects by direct binding of NF-κB complex to the glucocorticoid receptor.[118] Moreover, gene expression profiling identified NF-κB pathway activation to be correlated with treatment relapse in T-ALL.[119]

Several alterations in the apoptotic pathway itself have been described in pediatric leukemia, including mutations in tumor suppressor protein p53[120,121] in ALL and upregulation of the anti-apoptotic protein BCL-2 itself or one of its family members in ALL[121-129] as well as in AML.[130-138]

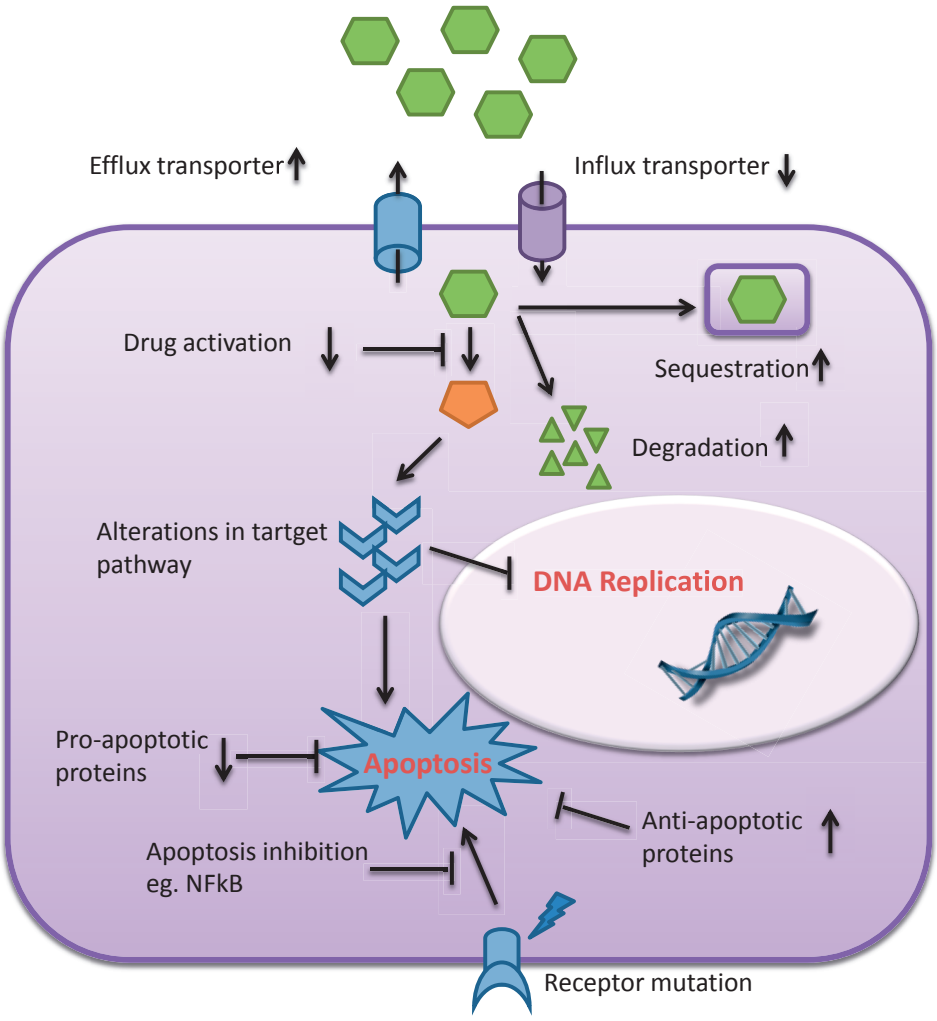

Figure 3. Overview of drug resistance mechanisms in acute leukemia.

EXPERIMENTAL THERAPEUTICS

Despite the substantial increase in survival, still a 20-25% of children with ALL and 30-40% of the pediatric AML patients are confronted with a relapse.[6,22,48] In addition, leukemia treatment consists of intensive courses of multiple chemotherapeutics with a variety of long-term side effects. To this end, new classes of effective drugs with diminished short- as well as long-term side effects are needed in order to make a substantial step forward in improving treatment outcome. In this thesis we focus on proteasome inhibitors, both the founding member bortezomib (BTZ) and new generation proteasome inhibitors, as a novel class of promising anti-leukemia drugs that might fulfill those criteria.

The proteasome is a large intracellular protease that consists of a core catalytic complex and two regulatory subunits. The core complex harbours, among other subunits, the catalytic β-1, -2, and -5 subunits which contain the postglutamyl peptidyl hydrolytic-, tryptic-, and chymotryptic-like proteolytic activities, respectively. The ubiquitin-conjugating system targets proteins for degradation by attachment of poly-ubiquitin (Ub) chains after which they are degraded by the proteasome. More than 80% of all eukaryotic protein degradation is controlled by this ubiquitin-proteasome pathway. When the proteasome is inhibited, misfolded and poly-ubiquitinated proteins accumulate thereby causing cytotoxic effects. Moreover, inhibition of multiple pro-survival pathways contribute to the proteasome inhibitor induced apoptosis. BTZ reversibly inhibits the chymotrypsin-like activity of the β5 subunit and to a lesser extent the β1 subunit of the proteasome leading to blockage of proteasomal degradation of ubiquitinated proteins.

At the time of start of this thesis project, BTZ had shown promising results in initial preclinical and clinical studies, either as single agent or in combination with other anti-leukemic drugs. The majority of these studies were performed in multiple myeloma (MM) and lymphoma. Therefore further exploration in a leukemia setting was warranted. Moreover, at the beginning of this thesis project, few studies had been performed addressing the question of resistance to proteasome inhibitors. The conventional mechanism of drug resistance mediated by efflux pumps, seemed to play only a modest role.[139,140] In contrast, the β-subunit composition was correlated with sensitivity and resistance to BTZ.[141,142] In addition several transcription factors and heat shock proteins were opted to be involved in BTZ resistance.[143,144] Further research was necessary to understand mechanism of acquired and intrinsic resistance to BTZ in leukemia.

THESIS AIM AND INTRODUCTION TO THE CHAPTERS

This thesis aims to explore the potential benefit of proteasome inhibitors as a new anti-leukemic treatment modality by addressing three main research objectives:

- Determine whether proteasome inhibition by BTZ and new generation proteasome inhibitors display anti-leukemic activity against pediatric leukemia cells *ex vivo*.
- Unravel molecular mechanisms of acquired resistance to BTZ after prolonged *in vitro* exposure of leukemia cell lines to this drug, and indentify strategies to overcome BTZ resistance.
- Identify determinants that can predict BTZ sensitivity in order to select patients eligible for proteasome inhibitor-based treatment.

Following this general introduction to leukemia and current treatment options, **chapter 2** covers a review that summarizes the original rationale for targeting the proteasome for therapeutic interventions in leukemia. This review also discusses initial preclinical and early clinical studies with BTZ as the prototypical proteasome inhibitor, either as single agent or in combination with other anti-leukemic drugs.

In **chapter 3**, we evaluate in an *ex vivo* setting the anti-leukemic activity of several proteasome inhibitors against primary pediatric ALL and AML samples. Analysis included BTZ and new generation proteasome inhibitors including irreversible proteasome inhibitors and proteasome inhibitors that specifically target the immunoproteasome rather than the constitutive proteasome. Furthermore, these studies were extended to examine whether differential expression levels of immunoproteasome versus constitutive proteasome in leukemia cells may serve as a novel marker to predict response to proteasome inhibitors.

The dynamics of emergence of acquired resistance to BTZ was investigated for AML cells (**chapter 4**), and ALL and MM cells (**chapter 5**), following chronic exposure to stepwise increasing concentrations of this drug. We identified mutations in a highly conserved BTZ binding pocket of the PSMB5 gene together with upregulation of the β5 subunit of the proteasome as underlying mechanism of BTZ resistance in these leukemia cells. In addition we explored strategies to overcome BTZ resistance by new generation proteasome inhibitors and other experimental drugs.

Finally, **chapter 6** reports on in-depth analyses of gene expression profiling of BTZ-sensitive versus BTZ-resistant ALL cells. Myristoylated Alanine Rich C Kinase Substrate (MARCKS) gene, appeared to be markedly and differentially overexpressed in resistant cells and was examined in a larger series of BTZ-resistant leukemia cell lines and primary leukemia samples. The protein encoded by this gene could be assigned a role in facilitating vesicular extrusion of ubiquitinated proteins, thereby contributing to BTZ resistance.

Summary, general discussion and future perspectives section (**chapter 7**) covers an integration of all data described in this thesis to put them in a perspective for improved exploitation of proteasome inhibitors in a (pediatric) leukemia setting followed by a thesis layman's summary of the main findings of this thesis in Dutch.

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