Molecular mechanisms of bortezomib resistance in acute leukemia

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Chapter 7

Summary, general discussion and future perspectives

SUMMARY

In this thesis, we investigated the possible application of bortezomib (BTZ), the first clinically available proteasome inhibitor, in the treatment of acute leukemias. In our studies we aimed to determine whether proteasome inhibition by BTZ along with other new generation proteasome inhibitors confers anti-leukemic activity against pediatric leukemia cells *ex vivo*. Since acquired resistance is an emerging problem during BTZ treatment in Multiple Myeloma (MM) patients, we developed BTZ-resistant leukemia cell line models to unravel the underlying molecular mechanism(s) of acquired resistance to BTZ. Moreover, we aimed to identify (bio)markers that can assist to identify and/or predict clinical BTZ resistance, thus helping in the selection of patients being eligible for BTZ treatment.

Chapter 1 describes the current status of knowledge on the genetic background of acute leukemia and discusses current treatment modalities. Of all pediatric malignancies, acute leukemia, consisting of Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML), is the most common type of childhood cancer. In adults, acute leukemias represent a much smaller fraction of all malignancies seen in this age group.

With a 5 year overall survival (OS) 83-94% the prognosis of pediatric ALL is considerably better as compared to adults (OS 15-35%, dependent on the age of the studied adult population). Similar difference in prognosis between children and adults is seen AML with a 5 years OS of respectively 65%-70% and 10-45% (dependant on the age of the studied adult population).

Currently both types of leukemia are treated with a combination of heavy chemotherapeutics. Despite this aggressive regime, still a significant subgroup of the leukemia patients relapses. In addition, leukemia survivors suffer from both short term as well as long term side effects. Therefore, new treatment options are needed to improve the outcome of these hematological malignancies and reduce the frequently occurring side effects.

Chapter 2 covers a review that summarizes the original rationale for targeting the proteasome for therapeutic interventions in leukemia. The proteasome is a large high molecular weight intracellular protease that consists of a core catalytic complex and two regulatory subunits. The core proteasome complex is made up of four stacked rings. The two outer rings contain seven α-subunits, while the two inner rings consist of seven β-subunits. Of these, the β1, β2, and β5 subunits contain the postglutamyl peptidyl hydrolytic-, tryptic-, and chymotryptic-like proteolytic activities of the proteasome, respectively. Together, for proteins to be degraded, these three subunits can hydrolyze almost all peptide bonds into smaller polypeptide units which can be further degraded by aminopeptidases. Prior to proteasomal degradation, proteins are first tagged with

poly-ubiquitin chains to be recognized by the ubiquitin-proteasome pathway, which controls more than 80% of all eukaryotic protein degradation.

When the proteasome is inhibited, misfolded and poly-ubiquitinated proteins accumulate intracellularly thereby causing cytotoxic effects. Additionally, inhibition of multiple pro-survival pathways contributes to a proteasome inhibitor-induced apoptosis. BTZ reversibly inhibits the chymotrypsin-like activity of the β5 subunit and to a lesser extent the caspase-like activity of the β1 subunit of the proteasome, leading to a blockade of proteasomal degradation of ubiquitinated proteins. This review also discusses promising initial preclinical and early clinical studies with BTZ, either as single agent or in combination with other anti-leukemic drugs. In addition, second generations of proteasome inhibitors, designed to overcome BTZ-induced side effects and BTZ resistance, are covered.

In **chapter 3**, we evaluated the *ex vivo* sensitivity of pediatric leukemia cells for BTZ in comparison to 3 next generation proteasome inhibitors: the epoxyketone-based irreversible proteasome inhibitors carfilzomib, its orally bio-available analog ONX 0912, and the immunoproteasome inhibitor ONX 0914. In addition, possible synergy of dexamethasone with BTZ was determined. We showed that ALL cells were up to 5.5-fold more sensitive to proteasome inhibitors than AML cells ($P<0.001$) and the combination of BTZ and dexamethasone was additive to synergistic in the majority of patient specimens. When the protein expression levels of the catalytically active β-subunits of the immuno- and constitutive proteasome were correlated to proteasome inhibitor sensitivity, both ALL and AML showed that increased ratios of β5i/β5, β1i/β1 and β2i/β2 correlated with increased sensitivity to proteasome inhibitors. These data suggest that differential composition of proteasomal β-subunits in acute leukemia cells is a contributing factor underlying BTZ sensitivity and resistance.

The dynamics of emergence of acquired resistance to BTZ was investigated in the AML cell line model THP-1 (**chapter 4**), the ALL cell line model CCRF-CEM, and the MM cell line model RPMI-8226 (**chapter 5**), following chronic exposure to stepwise increasing concentrations of this drug. The resistant cells showed marked cross-resistance to other proteasome inhibitors, but not to classic cytostatics, indicating a proteasome-specific resistance mechanism. When the β-subunit expression was evaluated, a markedly increased expression of constitutive subunits, predominantly β5, was observed, resulting in a decreased immuno-/ constitutive proteasome ratio. Moreover, using native gel electrophoresis, we showed that increased constitutive β-subunits were incorporated in the proteasomal complex. Mutation analysis of exon 2 of the PSMB5 gene, encoding for that part of the β5-subunit protein where BTZ primarily binds, identified several point mutations resulting in amino acid substitutions within the highly conserved BTZ binding pocket. Together, the genetic alterations in the BTZ-resistant cell lines formed a mutation cluster region in the BTZ-binding pocket of the β5 subunit, in particular the

S1 specificity pocket. In **chapter 5** *in silico* binding analyses showed decreased binding of BTZ to the different mutated β5 variants due to impaired direct BTZ interaction with, or by conformational changes of, the binding pocket. Consistently, modeling of the β5 substrate LLVY-AMC into binding pocket revealed a decreased binding affinity as well. Thus, β5 mutations have a concomitant impact on the hydrolyzing capacity of peptide substrates. These results were corroborated by using in gel β5 activity assays. Proteasome subunit overexpression is therefore an essential compensatory mechanism for the impaired catalytic activity of these mutant proteasomes.

Finally, in **chapter 6**, we report the results of multi-modality (DNA, mRNA, miRNA) array-based analysis of human CCRF-CEM leukemia cells and two BTZ-resistant subclones to determine whether besides β5 mutations other complementary mechanisms contribute to BTZ resistance. These studies revealed signatures of markedly reduced expression of proteolytic stress related genes in drug resistant cells over a broad range of BTZ concentrations along with a highly upregulated expression of the myristoylated alanine-rich C-kinase substrate (MARCKS) gene. MARCKS upregulation was not only noted in BTZ-resistant leukemia cell lines, but also in BTZ-resistant solid tumor cell lines and leukemia cells with acquired resistance to other proteasome inhibitors. Moreover, of potential clinical interest, when MARCKS protein expression was examined in clinical specimens derived from therapy-refractory pediatric leukemia patients (n=44), higher MARCKS protein expression trended ($p=0.073$) towards a dismal response to BTZ-containing chemotherapy. Mechanistically, we showed a BTZ concentration-dependent association of MARCKS protein levels with the emergence of ubiquitin-containing vesicles in resistant cells both in the CEM cell line model as well as in two ALL primary patient samples. These vesicles were found to be extruded and taken up in co-cultures with proteasome-proficient acceptor cells. Collectively, we propose a novel mechanism of BTZ resistance via exocytosis of ubiquitinated proteins in BTZ-resistant cells leading to quenching of proteolytic stress, and wherein MARCKS may function as a candidate marker protein for BTZ resistance.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In the studies described in this thesis, we aimed to investigate the anti-tumor efficacy and resistance of proteasome inhibition in leukemia in a preclinical setting. First, we determined the *ex vivo* efficacy of proteasome inhibition in pediatric leukemic samples (1). Furthermore, we developed several bortezomib (BTZ) resistant cell lines in order to unravel the underlying mechanism of acquired BTZ resistance (2). Finally, we used primary pediatric patient acute leukemia samples in combination with BTZ resistant cell line models to identify possible novel determinants that might aid selecting patients eligible for proteasome inhibitor treatment (3).

Ex vivo efficacy of multiple proteasome inhibitors in pediatric leukemia

At the time of the start of the studies, very limited clinical data and no clinical samples were available from BTZ-treated (pediatric) leukemia patients. Therefore, we started our studies with exposing untreated primary AML and ALL samples *ex vivo* to BTZ to determine their differential sensitivity/resistance profiles. We showed **(chapter 3)** that nanomolar drug concentrations ranges of BTZ can induce *ex vivo* cytotoxicity in a considerable cohort acute pediatric leukemias, thereby confirming data reported in small groups of pediatric patient samples [1-3].

As for many chemotherapeutics, ALL patients were more sensitive to BTZ as compared to AML patients. These data are concurrent with the results of Szczepanek and colleagues who showed that pediatric AML samples were relatively more resistant to BTZ[3]. Interestingly, in our study both T-ALL and pro-B-ALL cells were sensitive to BTZ, while Szczepanek *et al.* observed that T-ALL was even more sensitive to BTZ than B-ALL[3]. The latter finding might be explained by the ability of BTZ to inhibit the Notch1 pathway, known to be important in the pathogenesis of T-ALL[4]. Since T-cell leukemia has in general a worse prognosis as compared to B-cell leukemia[5], this subgroup in particular might benefit from BTZ treatment.

After the successful introduction of the reversible proteasome inhibitor BTZ, second generation proteasome inhibitors were designed[6,7]. In contrast to BTZ, which reversibly inhibits the chymotrypsin-like as well as the caspase-like activity, the second generation proteasome inhibitor carfilzomib selectively and solely inhibits chymotrypsin-like activity in an irreversible manner[8]. Carfilzomib has demonstrated potent pre-clinically potent in MM[8] and Waldenstrom Macroglobulinemia (WM)[9], and is currently showing activity in a clinical setting [10,11]. The first clinical results suggest that carfilzomib might be active in patients who relapsed or were refractory under BTZ treatment[12]. An orally available formulation of carfilzomib, i.e. ONX 0912 (PR-047, oprozomib), has also been reported to be pre-clinically active in MM[13]and WM[14]. Our studies were the first to show cytotoxic efficacy of carfilzomib and ONX 0912 in primary pediatric leukemic cells with a

comparable sensitivity profile as BTZ in pediatric ALL and AML. Recently, these findings were confirmed in primitive CD34+ primary patient samples[15]. Moreover, modest anti-leukemic activity was seen in a phase 1 trial in relapsed/refractory AML or ALL[16].

Since in hematological cells the immunoproteasome is predominantly expressed over constitutive proteasomes[17-19], specific targeting of this immuno variant of the proteasome was thought to elicit similar anti-leukemia effects and diminish toxic side effects as compared to conventional proteasome inhibitors. ONX 0914 (PR-957) is the first representative drug in the class of selective immunoproteasome inhibitors[20]. Using this compound we showed *ex vivo* anti-leukemia activity in the nanomolar concentration range analogous to the other proteasome inhibitors, thus indicating that immunoproteasome inhibition might be a clinical interesting targeting strategy for both ALL and AML.

The corticosteroid prednisone was the first effective drug in the treatment of ALL[21] and this class of drugs is still the cornerstone of current ALL protocols. In the current Dutch pediatric ALL protocol, impaired response to prednisone at day 8 of the treatment represents an important indicator of poor prognosis and is used in further risk stratification[22]. New drugs that are able to augment the glucocorticoid effect would be a valuable addition to the ALL treatment. When BTZ was combined with the corticosteroid dexamethasone, it showed additive/synergistic activity, particularly in the relatively dexamethasone-resistant patient samples. This implies that BTZ is a potentially promising new drug that can have additional effects when added to an AML treatment protocol, especially to a corticosteroid containing ALL treatment protocol.

Taken together, these *ex vivo* data of the tested proteasome inhibitors support further clinical investigation in leukemia. Horton *et al.* investigated in the feasibility of BTZ in pediatric patient with refractory acute leukemia[23]. No objective clinical responses were observed after BTZ monotherapy, scince the design of the study was phase I. Many clinical studies are currently ongoing, and the first clinical data on combination of BTZ with other chemotherapy in pediatric leukemias is now emerging and will be discussed in the future perspectives paragraph below. In the upcoming years, these studies will clarify whether or not our encouraging pre-clinical results will be corroborated in the clinic.

Resistance to proteasome inhibitors using *in vitro* cell line models

As with any new treatment strategy, selection of patients that will benefit from the treatment is essential. Since initially no leukemia samples were available from BTZ-therapy refractory patients, we developed an *in vitro* BTZ resistance model in human leukemic cell line models to explore possible underlying mechanisms of resistance. Using stepwise increasing concentrations of BTZ over a period of multiple weeks to several months, we were able to isolate BTZ-resistant cell lines of acute monocytic leukemia THP-1 cells **(chapter 4)**, T-ALL CCRF-CEM cells and RPMI-8226 multiple myeloma (MM) cells **(chap-**

ter 5). Intriguingly, the CCRF-CEM cells achieved resistance to 200 nM BTZ within a few weeks, THP-1 cells needed some additional weeks to obtain resistance to 200 nM BTZ, while it took several months to thrive 8226 cells resistant to 100nM BTZ. Whether these differential profiles in acquired resistance to BTZ is also clinically tumor type dependent (e.g. related to chromosomal instability), is yet to be determined.

By comparing the stably resistant cell lines with their parental sensitive cell lines we could identify several parameters related to BTZ resistance.

Upregulation of proteasomal subunits

Upregulation of the primary drug target is a common response upon acquisition of drug resistance, which was also observed as an initial alterations in the BTZ-resistant leukemia cell lines. Since our BTZ-resistant leukemia lines were stably resistant to BTZ for more than 6 months in the absence of drug, this pointed to irreversible genetic alterations. Indeed, chromosomal copy number analysis of the BTZ-resistant cell lines showed amplification of the *PSMB5* gene in CEM/BTZ, THP-1/BTZ as well as in 8226/BTZ. Interestingly, although the resistant cells showed variability in the size and extent of the amplification of chromosome 6, they all showed amplification of at least the *PSMB5* gene. PSMB5 mRNA levels were not highly upregulated, protein levels, however, were markedly increased in all BTZ-resistant cell lines. Upregulation of proteasomal subunits, and the β5 subunit in particular, has now been identified as a common feature in basically all published BTZ-resistant cell lines (reviewed in Kale[7,24]), hence confirming its role in BTZ resistance.

Initially we hypothesized that the abundant β5 subunit would not be assembled into the proteasome but could possibly function as a BTZ scavenger protecting the proteasome for BTZ inhibition. This hypothesis was disproved by gel filtration and native gel experiments, showing no free β5 subunits and upregulation of β5 incorporated in the proteasome (**Chapter 4**). While the BTZ-resistant CEM subline showed upregulation of the whole proteasome, BTZ-resistant 8226 cells featured a shift in composition from immunoproteasomes (particularly β5i expression) to constitutive β5 proteasome expression. Both changes resulted in a net increased expression of constitutive proteasome and a decreased immuno- / constitutive proteasome ratio.

Alterations in subunit composition of the proteasome has been linked to BTZ sensitivity by other research groups as well. BTZ-resistant Burkitt lymphoma (Namalwa) cells also showed a shift from immunoproteasome towards constitutive proteasome[25]. Similarly, BTZ-resistant AML cells were characterized by a decreased immuno- / constitutive proteasome ratio[26]. Busse *et al.* showed that intrinsic BTZ-resistant solid tumor cell lines express lower levels of immunoproteasome subunits as compared to the more sensitive hematologic B-cell lines. Interferon gamma (IFN-γ) pretreatment, which induces β5i and replaces constitutive β5, enhanced sensitivity to BTZ in 50% of the tumor cell lines[27].

Niewerth *et al.* confirmed these findings for our BTZ-resistant cell lines (CEM, THP1 and 8226). These lines showed β5i upregulation together with increased BTZ sensitivity (up to 7-fold) after INF-γ exposure[28]. All together these data imply the importance of the proteasome composition in BTZ sensitivity and resistance.

Mutations in the β5 subunit

Mutations in cellular enzymes targeted by chemotherapeutic drugs is also a well-established modality of drug resistance[29-32]. Analysis of the proteasomal subunits of the BTZ resistant cell lines identified several mutations in the β5 subunit of the proteasome as well. Notably, point mutations were identified in exon 2 of the *PSMB5* gene encoding part of the β5 subunit protein harboring the BTZ binding pocket. This highly conserved region seems to be a mutation "hot spot" when cells are exposed to BTZ for a prolonged period. In fact, several different *in vitro* tumor models showed mutations in this region upon acquisition of BTZ resistance[7]. Figure 1 shows an updated summary of all mutations found in BTZ resistant cell lines, including non-hematological malignancies. All mutations result in amino-acid alterations in, or in close vicinity of, the BTZ binding pocket[33]. Except for the A247G mutation which results in Thr21Ala substitution, all of the mutations result in amino-acid alterations in de S1 pocket of the β5 subunit. This specificity pocket is a highly conserved part of the subunit, which is mainly responsible for recognizing the peptide bond of the substrate. This is also the site that has to be cleaved and determines the specificity as well as facilitates the binding of the P1 side chain of BTZ[33,34]. Our *in silico* analysis in **chapter 5** provided evidence for hindered BTZ binding. The majority of the mutations resulted in substitution of amino acids which directly bind to BTZ. The only exceptions were the Met45Ile and Met45Val substitutions, which do not directly interact with BTZ. However, Met45 is known to contribute to

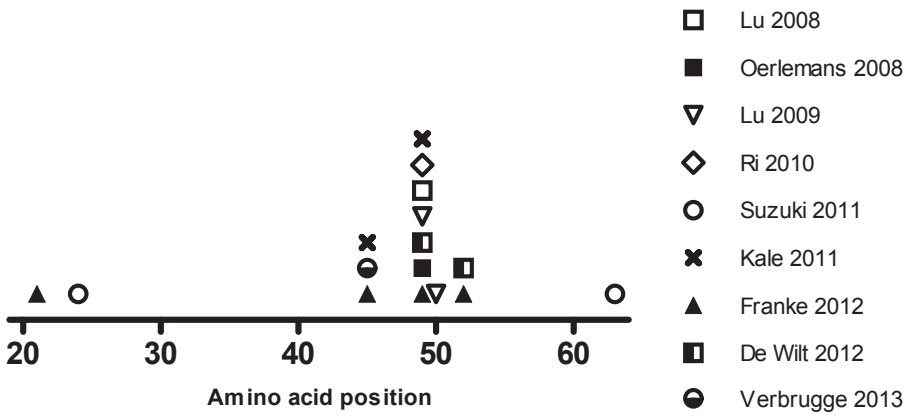

Figure 1. Summary of aminoacid substitutions within β5 subunit of the proteasome in BTZ resistant cell line models.

the specificity of the S1 pocket and upon binding, Met45 undergoes a conformational change and shifts the direction of its side chain toward Cys52 vicinity[33]. Alterations in this amino-acid might therefore hinder this conformational change and contribute as well to decreased BTZ binding.

Since the mutations are all located in the highly conserved substrate binding pocket, the question was addressed whether it also impacted the catalytic activity of the β5 subunit. Using the Suc-LLVY-AMC substrate in native gel electrophoresis analysis, we showed in the 8226/BTZ100 MM line (harboring the Thr49Ala substitution), that despite the upregulated expression of the β5 subunit, conversion of this substrate was substantially decreased. This was also noted in the 8226/BTZ7 cells (harboring the Ala21Thr substitution) albeit to lesser extent. These BTZ-resistant 8226 cell lines almost exclusively expressed the mutated constitutive β5[28]. In contrast, the CEM/BTZ7 (harboring the Cys52Phe substitution) and CEM/BT200 (harboring both the Cys52Phe and Ala49Val substitution) showed an increased degradation of the Suc-LLVY-AMC substrate, which may be explained by the increased expression of both the β5 (non-mutated) and β5i subunits in these resistant cells. The data from the 8226 BTZ-resistant cell lines suggest that the decreased Suc-LLVY-AMC substrate degradation in the 8226/BTZ7 and 8226/BTZ100 is linked to the Ala21Thr and Ala49Thr substitution, respectively. Docking the LLVY-AMC substrate *in silico* in the crystal structure of the β5 subunit supported the hypothesis of decreased binding of the substrate in the mutated binding pocket.

Our data are concurrent with results by others for BTZ-resistant cells. Characterizing BTZ-resistant lung cancer cells, De Wilt *et al*. showed upregulation and mutation of the constitutive β5 subunit with decreased conversion of the suc-LLVY-AMC substrate on native gels[35]. Not surprisingly, intrinsic expression levels of β5i were low both in parental lung cancer cells and the BTZ-resistant cell lines. Verbrugge *et al.* investigated BTZ resistance in the B-cell JY cell line model in which a mutation leading to an amino acid substitution (Met45Ile) in the β5 subunit of the proteasome was identified[36]. Although no proteasomal activity measurement was performed, their model showed a distinct upregulation of the constitutive β5 subunit in the BTZ-resistant clone. In addition, Suzuki *et al.*, who identified Cys63Phe and Arg24Cys substitution in a BTZ-resistant adenocarcinoma cell line model, showed upregulation of the constitutive and immune β5 subunits in the resistant lines but without increased conversion of chymotrypsin activity[37]. Notably, recovery time of proteasomal activity in the resistant cell lines after drug removal was significantly decreased. Ri *et al.* identified the Ala49Thr amino acid substitution in the β5 subunit in a BTZ-resistant MM cell line model [38]. In contrast to our results, they showed a slightly increased suc-LLVY-AMC conversion in the BTZ-resistant cell lines when exposed to 10 nM BTZ. Also Lu *et al.* identified the Ala49Thr amino acid substitution and the Ala50Val substitution in an ALL cell line models resistant to BTZ[39,40]. Also these researchers showed decreased inhibition of the chymotrypsin activity (as

measured by suc-LLVY-AMC conversion) in BTZ resistant cell lines, being most pronounced in the cell line resistant to 1000 nM of BTZ. Additionally they showed a slightly decreased inhibition of the chymotrypsin activity after BTZ exposure when the WT cell line was transfected with a mutant *PSMB5* gene. Apparent differences with respect to impact of *PSMB5* mutations on catalytic activity might be explained by the different methodologies for chymotrypsin activity measurements. While we used activity on native gel, showing activity on proteasomal level, other groups made use of a intact cell-based assays[38] or whole cell lysate[39,40] analysis.

The hypothesis that a mutation in the *PSMB5* gene leads to resistance to proteasome inhibitors, was further supported by findings in an unexpected area of science, i.e. marine biology. Kale *et al.* sequenced the proteasomal subunits of the *S.tropica* which produces the proteasome inhibitor salinosporamide A (NPI-0052, marizomib)[41]. They identified critical changes in the β5 homologue of the S. tropica at amino acid position 45 and 49 as compared to other actinobacteria, i.e. Met45Phe and Ala49Val, which conferred 'self-resistance' to Salinosporamide A in S.tropica. Moreover, they showed that *S.tropica*, as well as the actinobacterium proteasome introduced with the indicated amino acid alterations, had a decreased conversion of the suc-LLVY-AMC substrate. These activity results are in line with data of an Ala49Val substitution in yeast. Mutations leading to this substitution resulted in reduced chymotrypsin like activity[42].

Evidence that β5 subunit mutations also extend to acquired resistance to second generation proteasome inhibitors was recently provided by Niewerth *et al.*[43,44] They showed that prolonged exposure towards the immunoproteasome inhibitor in PR-924 to CCRF-CEM, THP-1 and RPMI-8226 cell lines resulted in resistance development due to a mutation in the *PSMB5* gene translating respectively into Met45Ile, Ala49Thr and Met45Val amino acid substitution in the resistant cell lines. Finally , acquired resistance to salinosporamide A in CCRF-CEM cells was also accompanied with an amino acid substitution (Met45Val) in the β5 subunit[43]. Importantly, all leukemia cell lines resistant to second generation proteasome inhibitors displayed cross resistance towards BTZ, albeit to a lower extent than cells primary selected for BTZ resistance.

The impact of β5 mutations for BTZ resistance were recently corroborated by studies from Huber et al.[45] In a yeast model system, mutations were introduced in the psmb5 gene. Subsequently cell growth, proteasome inhibition, and X-ray crystallography analyses were performed. With exception of Thr21Ala, their panel mutations included all proteasomal alterations found in our BTZ resistant hematological cell line models and showed both resistance to proteasome inhibitors as well as decreased turnover of proteasomal substrates.

Taken together, these data provide solid evidence for the role of mutations in the β5 subunit in conferring proteasome inhibitor resistance *in vitro*. This resistance modality may partly be overcome by the sensitizing effect of interferon gamma, which induces

(non-mutated) β5i expression and replaces and suppresses expression of the constitutive (mutated) β5 subunit[28]. Although the effect of the mutations on proteasome catalytic activity varies among different studies, all data obtained by purified proteasome, *in gel* proteasome activity analysis and yeast model show decreased chymotrypsin-like activity or least altered substrate specificity of the chymotrypsin-like activity.

Alternative protein disposal

Since drug resistance in general is typically a multifactorial mechanism, we performed multimodality array based screenings to identify possible additional resistance features. Gene expression profiling identified Myristoylated alanine-rich C-kinase substrate (MARCKS) as being highly upregulated in the BTZ-resistant CEM clones. This upregulation was not only confirmed on protein level in the BTZ resistant CEM, but also in the BTZ resistant THP-1. In addition, THP-1 and CEM cells resistant to other proteasome inhibitors, showed this MARCKS upregulation as well.

MARCKS is an 87 kDa protein that is involved multiple exocytosis processes including mucin secretion in airway epithelial mucosa[46], catecholamines exocytosis in chromaffin cells[47], large core vesicle exocytosis[48], mast cell degranulation[49] and degranulation in human leukocytes[50]. Therefore we propose a novel model for BTZ resistance in which misfolded ubiquitinated proteins are secreted in a MARCKS-dependent exocytosis process. We showed increased vesicle formation in BTZ-resistant CEM cells and their secretion in the extracellular medium along with uptake of the vesicles by HeLa cells. Moreover, we could clearly show co-localization of MARCKS with ubiquitin in these vesicles structures. These findings were corroborated in two primary leukemic patient samples; upon exposure to BTZ, cells from a BTZ-resistant patient revealed markedly increased formation of ubiquitin-containing vesicles as compared to cells from a BTZ-sensitive patient. The phenomenon of exosomes that contain poly-ubiquitinated proteins has previously been described by Buschow *et al.*[51]. They showed exosomes relatively enriched for ubiquitinated proteins as compared to total cell lysates indicating that ubiquitinated cytoplasmic proteins can selectively be incorporated into the multivesicular body pathway. Pérez-Galán *et al.* showed that BTZ-resistant Mantle Cell Lymphoma (MCL) cells gain a plasmacytic like phenotype, reminiscent of secretory cells. Although the BTZ-resistant MCL cells did not produce immunoglobulins[52], this was not investigated for secretion of ubiquitinated proteins. Future experiments should be dedicated to identify; (a) the mechanism of vesicle formation in BTZ-resistant cells, (b) the nature of the vesicles (exosomes, others) and (c) whether MARCKS has other partner proteins that facilitate exocytosis of ubiquitin-containing vesicles as a mechanism of BTZ resistance.

Conceivably, the concept of alternative protein disposal is a logical bypass for the endoplasmatic reticulum(ER) stress induced by proteasome inhibition. In line with this, we found evidence for the lack of ER stress-related gene expression in the BTZ-resistant

cells. Since BTZ sensitivity is determined by the balance between protein load and proteasome capacity[53], secretion of poly-ubiquitinated proteins will diminish the protein load and consequently BTZ sensitivity.

A well-known mechanism for alternative protein disposal is autophagy. This process has been linked to BTZ resistance in several tumor models[54-60]. Inhibition of autophagy through different mechanisms including calpain inhibitor[55], downregulation of Heat Shock Protein B8 (HSPB8)[56], B-cell lymphoma 2 interacting mediator of cell death (BIM) upregulation[59], Tipifarnib[54] or (hydroxy)chloroquine[61,62] increased BTZ sensitivity in a MM model. In addition, histone deacetylase (HDAC) inhibitors can block autophagy by disrupting aggresome formation, the process that precedes autophagy[63,64]. Despite the potential impact of autophagy on BTZ resistance, no evidence for autophagy involvement was indicated in BTZ-resistant CEM cells as GEP studies showed no upregulation of autophagy related proteins (including HSPB8) and even a downregulation of the autophagy initiator SQSTM1.

Altogether, in CCRF-CEM and primary leukemia cells we identified multiple factors contributing to a BTZ resistant phenotype, including alterations in the immunoproteaseome/constitutive proteasome (subunit) composition, point mutations in the β5 subunits, and overcoming of proteolytic stress by exocytosis of ubiquitinated proteins.

Determinants for clinical response to proteasome inhibition

Although *in vitro* models are a valuable tool to identify possible mechanisms of BTZ resistance, assessment of the relevance for the clinic requires validation in *ex vivo* studies using primary patients samples. Preferably this is examined in add-on studies of clinical trials that include BTZ in the treatment protocol.

Proteasome composition

Add-on studies of a clinical trial in MM showed a correlation between BTZ sensitivity and proteasomal expression levels in a cohort of 270 MM patients. Overexpression of proteasome genes, especially *PSMD4*, 48 hours after BTZ administration was correlated with worse prognosis[65]. In addition, in a short report, 1 BTZ resistant MM patient showed high *PSMB5* gene expression compared to 2 responding patients[66]. In contrast, lower proteasomal mRNA expression before BTZ treatment and upregulation of proteasomal mRNA expression after 6 and 24 hours after treatment correlated with clinical response in a cohort of 5 MCL patients[67].

Compared to MM, clinical data on BTZ response parameters in leukemia is limited. Matondo *et al.* reported in a small group primary AML samples a possible correlation between higher 20S protein expression and BTZ sensitivity[68], but no further analysis of the proteasome subunit composition was performed. To address this issue in further detail, we correlated the composition of the proteasome to *ex vivo* sensitivity for proteasome

inhibitor in a cohort of pediatric AML and ALL samples. We showed that increased *ex vivo* BTZ sensitivity of primary AML cells correlated with lower β5 expression. In fact, sensitivity for BTZ in AML was inversely correlated with higher ratios of β1i/β1 and β2i/β2 whereas a trend was noted for β5i/β5. ALL samples showed higher sensitivity to BTZ as compared to AML. This increased BTZ sensitivity in ALL cells also came with a higher β2i/β2 ratio and a trend of a higher β1i/β1 ratio, but did not correlate in a significant manner. Together, these data indicate that relative high immuno/constitutive proteasome ratio conduces an increased BTZ sensitivity. Very recently, these data were confirmed in AML and ALL samples obtained from two pediatric clinical COG trials (AAML07P1 and AALL07P1) in which BTZ treatment was incorporated, showing similar results[69]. Collectively, assessment of (immuno/constitutive) proteasome compositions may help to select leukemia patients who will respond to BTZ treatment and thereby personalize their treatment. Nowadays, several techniques, utilizing either Western blots[28], ELISA-based assays[8] or with activity-based probes[19] allow this type of analysis on clinical specimen.

Mutations in the β5 subunit in patients

The point mutations identified in *PSMB5* of BTZ-resistant cell line models, are distinct of *PSMB5* SNPs found in the general population. Wang *et al.* sequenced the *PSMB5* gene in a large cohort of healthy persons and 61 MM patients after BTZ treatment[70]. No SNPs were found in the exon 2 of the *PSMB5* gene neither in the general population nor in MM patients. They did find polymorphisms that influenced *PSMB5* gene expression, but these did not correlate with BTZ response. It must be noted that sequence analysis in one third of the MM patients was performed only on whole blood, not on isolated malignant plasma cells, which may have influenced the sensitivity of the analysis. In addition, it was not stated how many BTZ-resistant patients were included in the study. More recently, Lichter *et al.* sequenced the *PSMB5* gene in tumor samples of MM patients included in the APEX trial in which patients were treated with either BTZ or dexamethasone[71]; no mutation(s) were found in this group. Although the sample sizes in the indicated studies were limited, the data suggests that *PSBM5* mutations are not a common cause of acquired BTZ resistance in clinical MM treatment protocols. Whether this also holds true for leukemias or MM following long-term BTZ maintenance therapy, is yet to be determined. Currently, clinical samples are collected from the previously mentioned COG (AAML07P1 and AALL07P1) and European (NTR1881) studies, in order to answer this research question.

MARCKS expression in patients

In our pre-clinical BTZ resistant leukemia model, we found a marked upregulation of MARCKS. To test whether this upregulation might be a prognostic marker for BTZ resistance, we collected primary ALL patient samples obtained from the clinical COG trial AALL07P1 using combination chemotherapy including BTZ. A strong trend was

seen in the inverse correlation between MARCKS expression and clinical response. Since the samples were obtained in the setting of a clinical trial with combination therapy, a direct correlation between BTZ response and MARCKS expression cannot be made. However, our findings are further supported by Micallef *et al.* who showed MARCKS protein upregulation in a small group of BTZ resistant MM patients[72]. Consistently, Yang *et al.* identified MARCKS upregulation as well as an resistance marker in primary MM samples[73]. Interestingly, in their studies reversal of BTZ resistance could be achieved by inhibition of MARCKS phosphorylation or MARCKS knockdown. In contrast, these approaches were not effective in our leukemia cell lines models with acquired resistance to BTZ, conceivably due to dominance of *PSMB5* mutations.

To further assess the clinical relevance of MARCKS for BTZ efficacy in leukemia, we are prospectively validating the possible prognostic role of MARCKS in a pediatric ALL cohort treated with a combination BTZ with dexamethasone, vincristine and methotrexate (NTR1881)[74].

FUTURE PRE-CLINICAL PERSPECTIVES

Beyond the above-discussed mechanisms that contribute to BTZ resistance, other factors have been recognized to confer BTZ resistance, which are summarized in Figure 2. First, the level of stress response is crucial in BTZ-induced cytotoxicity. In this respect, it has been shown that low XBP-1 levels, a regulator unfolded protein response (UPR), predicts a poor response to BTZ, both *in vitro* and in MM patients[75]. In addition expression of ATF6, another regulator of the UPR, also correlated with BTZ sensitivity[76]. In MCL, low basal mRNA levels of oxidative stress pathway genes and their upregulation upon BTZ exposure correlated with *in vivo* response to BTZ [67]. Both studies in MM and MCL indicated that the absence of oxidative and proteolytic stress is a determinant for BTZ resistance. Second, interference with heat shock protein (HSP) function has been shown to increase BTZ-induced UPR and subsequent apoptosis induction[1,56,77-80], thus providing promising rationale for combination therapy of HSP-inhibitors and BTZ. Third, activation of several pro-survival pathways, including Nuclear Factor kappa B (NF-kB)[81-85], AKT/mTOR[86-88] and Insulin like growth factor 1 (IGF-1)[89-91], have been described to confer BTZ resistance. Since inhibition of these pathways elicits anti-cancer activity, further (pre) clinical research on their combination with BTZ warrants further evaluation. Lastly, the multidrug resistance efflux transporter MDR1/P-glycoprotein (P-gp) has been shown to contribute to BTZ resistance [92-94]. It should be mentioned, however, the substrate affinity of Pgp for BTZ is relatively poor as compared to second generation proteasome inhibitors such as carfilzomib[36]. Notwithstanding this fact, inhibition of P-gp may be considered as a strategy to improve the efficacy of BTZ and next generation proteasome inhibitors.

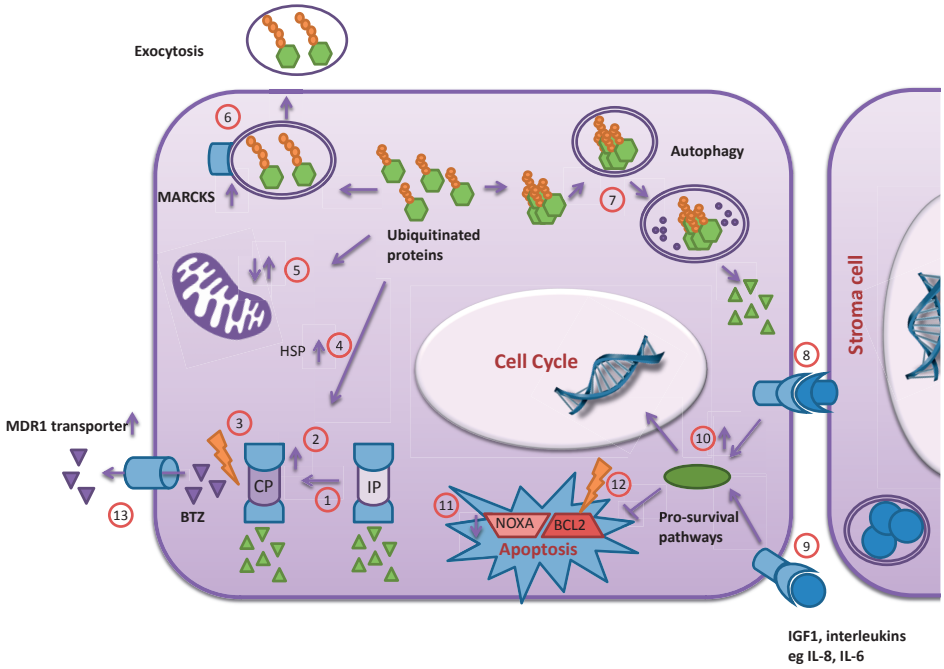

Figure 2. Overview of known molecular mechanisms involved in BTZ resistance. Proteasome related resistance: Relative down regulation of immunoproteasome as compared to constitutive proteasome (1) together with absolute upregulation of the constitutive proteasome (2) and mutation in the β5 subunit of the proteasome (3). Alternative stress handling: upregulation of heat shock proteins (4) or changes in redox metabolism(5) which can prevent oxidative stress. Alternative handling ubiquitinated protein: Exocytosis of ubiquitinated proteins in MARCKS-associated vesicles (6), and protein degradation through autophagy (7). Activation pro survival signalling: Intrinsic activation of pro-survival pathways e.g. AKT, NFκB or MET(8) or through stimulation by direct interaction with stromal cells (9) or indirectly through soluble growth factors or interleukins (10). Decreased apoptosis: Downregulation (11) or mutation (12) of pro-apoptotic proteins. Finally, extrusion of BTZ via multi drug resistance efflux pump MDR1/P-gp (minor effect, more pronounced in carfilzomib resistance) (13).

Current clinical progress using proteasome inhibitors in hematological malignancies

Although single drug treatment with BTZ did not show great clinical response in leukemia, combination with standard chemotherapy appeared more promising. The questions for the future would be to select the most effective drug combination with acceptable side effects and select the right patient population to achieve optimal benefit from a selected treatment regimen.

Combination strategies

For ALL, dexamethasone would be a good candidate drug to combine with BTZ. The combination has been shown to be effective in previously untreated MM[95] and we

showed *ex vivo* synergy of BTZ and dexamethasone in primary pediatric ALL samples. Although this combination was not clinically tested in ALL, when BTZ was combined with induction therapy including dexamethasone (DEX), vincristine (VCR), PEG-asparaginase (PEG-ASPA) and Doxorubicin (DOX) in relapsed pediatric ALL, promising CR rates were observed[96,97]. Currently, several clinical trials are ongoing in ALL, combining BTZ with several chemotherapy protocols which all include dexamethasone (See Chapter 2, Table IV). Notably, in one study (NTR1881) using BTZ, DEX, VCR and methotrexate (MTX) in relapsed pediatric ALL, the timing of BTZ administration in relation to DEX is investigated. This study should provide information on the optimal synergistic effects of the two drugs.

When the Pegylated liposomal DOX was combined with BTZ in hematological malignancies including MM, AML and NHL, again promising response rates were observed[98]. Addition of the anthracycline DOX to BTZ and DEX was also tested in newly diagnosed MM patients and resulted in a 90% response rate with well-tolerated and manageable toxicities[99]. In AML, idarubicin was combined with BTZ and little response was obeserved[100]. When cytarabine (Ara-C) was added, or when BTZ was combined with daunorubicin and Ara-C, response rate increased substantially [97,101]. Currently, several clinical trials are ongoing in AML, including anthracyclines and BTZ with other chemotherapy in the treatment protocols (See **Chapter 2**, Table IV). More information about possible synergy between anthracyclines and BTZ in AML might come from one of those studies in which BTZ is combined with DOX (NCT01736943).

Based on *in vitro* data, HSP inhibition seems a promising addition to BTZ treatment. Although some efficacy of the combination of the HSP90 inhibitor tanespimycin with BTZ was seen in relapsed or refractory MM[102], no effect of this combination was observed in a phase 1 trial in relapsed or refractory AML[97,103]. Since BTZ monotherapy also exerts limited clinical efficacy, adding BTZ and tanespimycin to AML protocols might increase clinical response. In addition new HSP90 inhibitors are upcoming, e.g. PF-04929113 (SNX5422) was recently tested in a phase I trial[104] and KW-2478 is being evaluated in combination with BTZ in MM in a phase I/II studies (NCT01063907). Moreover, HSP27 and HSP70 inhibitors are currently being evaluated pre-clinically (reviewed in Zhang *et al.*[105]). Additional clinical trials in leukemia are needed to evaluate a possible added value of the combination with BTZ.

HDAC inhibitors are a promising class of drugs to combine with BTZ. Since these inhibitors have a wide range of intracellular effects (reviewed in Hideshima *et al.*[106]), including an accumulation of ubiquitinated proteins through hyperacetylation of α-tubulin resulting in disruption of the transport of aggresomes to the lysosome, it is an interesting candidate for combination therapy with BTZ. Data from phase I studies in MM evaluating the pan-HDAC inhibitor panobinostat (LBH589) or vorinostat (Zolinza) in combination with BTZ have shown responses in patients who failed to respond previously to BTZ[107,108].

In leukemia, limited effects of mono-therapy with several HDAC inhibitors were seen in Phase I trials [109-113]. Currently, a combination of BTZ, vorinostat and the VEGF inhibitor sorafenib is investigated in poor risk AML (NCT01534260). Unfortunately, a clinical trial evaluating the combination of BTZ, vorinostat and DEX for relapsed or refractory ALL was terminated due to slow patient accrual (NCT01312818). More data in leukemia are needed to confirm the promising preclinical synergy of HDAC inhibitors with BTZ.

Targeting autophagy has pivotal effects on BTZ sensitivity. Increased autophagy has been linked to BTZ resistance in several tumor models[54-59] and combination of autophagy and proteasome inhibition by hydroxychloroquine and BTZ in a phase 1 trial in patients with relapsed/refractory myeloma showed synergistic effects[114]. Inhibition of mTOR by everolimus, a known autophagy inhibitor, in relapsed or refractory MM in a phase I/II trial showed some response as well[115]. In addition, clinical trials are ongoing combining the everolimus and BTZ in relapsed or refractory lymphoma (NCT00671112) or combining temsirolimus with BTZ in relapsed or refractory Non-Hodgkin lymphoma (NCT01281917). These data indicate that uncontrolled activation as well as inhibition of the autophagy pathway, might contribute to BZT sensitivity *in vivo*.

Second generation proteasome inhibitors

Despite the successful introduction of BTZ, several drawbacks such as resistance and toxic side-effects led to development of second generation proteasome inhibitors which are at several stages of clinical development. Due to promising preclinical studies, the irreversible proteasome inhibitor carfilzomib (Kyprolis, PR-171) has advanced rapidly into the clinic. Several trials of carfilzomib as a single drug or in combination therapy in initial or relapsed MM showed promising responses [116-122] even in MM patients not responding to BTZ regimes[123,124]. Currently, a phase III trial is comparing BTZ and DEX with carfilzomib and DEX in relapsed MM patients (NCT01568866). In addition, the drug is also tested in leukemia and lymphoma (NCT01137747 and NCT01212380). Our studies showed that the oral formulation of carfilzomib, oprozomib (ONX 0912) exerts *ex vivo* cytotoxic effects in primary pediatric leukemia samples (Chapter 3). This formulation is now investigated in clinical trials in several malignancies. Together, these data show that carfilzomib is a promising second generation proteasome inhibitor even for patients not responding to BTZ containing therapy.

Recently, an oral formulation resembling BTZ, ixazomib (MLN9708), has emerged into the clinic. This proteasome inhibitor showed preclinical activity in MM[125] and clinical activity in phase I studies in relapsed MM[126,127]. Several studies in MM are currently ongoing (www.clinicaltrials.gov). Moreover, two clinical trials investigating efficacy of ixazomib in AML are ongoing (NCT0230405 and NCT 02070458).

Two other oral proteasome inhibitors made their way into the clinic. Although delanzomib (CEP-18770), a boronic acid based proteasome inhibitor, showed preclinical

activity in MM[128] a phase II trial was prematurely terminated due to lack of efficacy[129]. The nonpeptic proteasome inhibitor marizomib (NPI-0052, Salinosporamide A) proved to be pre-clinically active in MM and leukemia cells [130,131]. Phase I studies with marizomib mono-therapy or in combination with the HDAC inhibitor vorinostat are just finished, data of these studies are not yet available. In addition, 2 phase I/II studies for marizomib are currently ingoing in relapsed MM(NCT02103335 and NCT00461045).

PR-957 (ONX 0914) and PR-924 represent members of a new class of proteasome inhibitors being directed specifically against the immunoproteasome[20,132]. PR-924 demonstrated pre-clinical efficacy in leukemia and MM[44,132]. In addition, in chapter 3 we showed ex vivo cytotoxicity of PR-957 in primary leukemia samples. Since leukemia cells, especially ALL cells, express high levels of immunoproteasome, it might be a good candidate for further clinical development in hematological malignancies.

Since Niewerth *et al.* showed development of acquired resistance to the second generation proteasome inhibitors marizomib and PR-924 [43,44], resistance issues have to be taken in account for second generation proteasome inhibitors as well. Therefore combination therapy will be still be needed for the treatment of leukemia in order to minimize/prevent resistance.

CONCLUSION

In this thesis we demonstrated preclinical efficacy of proteasome inhibitors in leukemia. In addition, we identified preclinical BTZ resistance mechanisms that are currently validated in several clinical trials. The combination of pre-clinical research and ongoing clinical studies will be needed to identify and confirm determinants of resistance and markers for clinical response in order to further personalize the treatment with proteasome inhibitors. Moreover, novel preferably orally available proteasome inhibitors with less short- and long-term side effects are warranted to further optimize the treatment efficacy. Finally, since in vitro results indicate that prolonged administration to second generation proteasome inhibitors comes with resistance development as well, evidence based combination studies will still be needed to achieve durable increased overall survival for leukemia patients.

KEY POINTS OF THIS THESIS

1. BTZ shows promising *ex vivo* activity in both pediatric acute lymphoblastic as well as myeloid leukemia.
2. Chronic *in vitro* BTZ exposure induces an unique proteasome inhibitor specific resistance mechanism that does not influence sensitivity to conventional chemotherapeutics.
3. Chronic BTZ stress results in multiple cell line models in mutations in the region of the PSMB5 gene that encodes for the part of the beta 5 subunit of the proteasome that directly binds to BTZ. This alteration causes decreased BTZ binding together with decreased substrate binding.
4. Besides mutations, BTZ-resistant cells show upregulation of the constitutive proteasome and a relative downregulation of the immunoproteasome, compensating for the decreased proteasomal function due to the mutation.
5. As a novel mechanism to decrease proteasomal load, BTZ resistant cells discard ubiquitinated proteins through vesicle-mediated exocytosis process.
6. MARCKS, a protein related to exocytosis, deserves further exploration as a possible clinical predictor of BTZ resistance in (pediatric) patients with acute leukemia.

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