Proteasome inhibitors in acute leukemia
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CHAPTER 9

General discussion and future perspectives
For treatment-refractory and relapsed acute leukemia patients with low chances of survival, there is an urgent need for improvement of therapeutic options. Proteasome inhibitors, particularly bortezomib, are being explored in the clinical setting in childhood acute leukemias, based on their proven track record in the treatment of several hematologic malignancies, especially patients with multiple myeloma (MM). This thesis therefore focused on exploring the potential role of proteasome inhibitor treatment for children with acute leukemia. Specifically, we aimed to answer the following questions: (i) can we identify which patients will benefit from bortezomib-containing therapy, (ii) what are the mechanisms underlying resistance to bortezomib, and (iii) can we find alternative treatment options for bortezomib-resistant patients. To this end, we first evaluated the efficacy of single agent bortezomib in leukemic cells, and combination treatment of bortezomib with conventional chemotherapeutics. Besides bortezomib, we also evaluated the application of next generation proteasome inhibitors, which can be immunoproteasome-specific or inhibit both constitutive- and immunoproteasomes. In addition, we studied the impact of long-term exposure of proteasome inhibitors and the generation of acquired resistance in cell lines. Lastly, we aimed to decipher the mechanisms of drug-resistance, a limiting factor for the efficacy of proteasome inhibitor treatment, and explored strategies to overcome bortezomib resistance.

MULTIPLE MYELOMA VERSUS ACUTE LEUKEMIA

Successful clinical activity of bortezomib in MM\(^1\) and lymphoma patients\(^2\) made its translation to leukemia an obvious choice, with currently 27 trials ongoing in acute leukemia patients. Data from clinical trials combining bortezomib with conventional chemotherapeutics published so far showed promising results in relapsed pediatric pre-B ALL patients, although the relevance for relapsed T-ALL and relapsed pediatric AML patients is still controversial\(^3\). Currently, the molecular basis for the response to bortezomib remains unclear. The good response of plasma cells to proteasome inhibitors has been attributed to their high protein turnover, in particular the balance between proteasome workload and catalytic capacity seems very important for the sensitivity of MM cells to bortezomib, since bortezomib-sensitive MM cell lines expressed lower proteasome levels but have a higher proteasomal degradation rate than relatively resistant MM cells\(^4\). Our data on proteasome composition in a MM cell line showed similar bortezomib-sensitivity and similar proteasome expression per ug total protein as acute leukemia cell lines. Notably, these cell lines were outbalanced in more constitutive proteasomes than immunoproteasome composition. In direct comparison, total proteasome amount in pediatric ALL and AML patient cells was similar to cell lines but differed in composition from cell lines by the fact that in patient cells the proteasome consisted for >70% of immunoproteasomes. This was also noted by Parlati et al.\(^5\) for other cells of hematologic origin, showing that CD138+ cells of MM patients and healthy volunteers were predominantly composed of immunoproteasomes. It should be emphasized that cell lines cannot be directly compared to patient samples in all respects.
BORTEZOMIB VERSUS NEXT GENERATION PROTEASOME INHIBITORS

Relevant clinical shortcomings of bortezomib relate to its unsuitability for oral administration, its toxic side effects such as peripheral neuropathy and the emergence of resistance. Toxicity can be diminished when bortezomib is administered in more optimized schedules, lowering concentration of frequency and changing the route of administration (subcutaneous instead of intravenous). Moreover, recently next generation proteasome inhibitors devoid of toxicities noted by bortezomib have been introduced. Furthermore, considering the abundant expression and functional relevance of the immunoproteasome in leukemic cells, novel opportunities for selective targeting of the immunoproteasome has gained considerable interest. In chapter 3 we compared the antileukemic efficacy of bortezomib to that of a series of next generation proteasome inhibitors: the irreversibly binding inhibitors carfilzomib (Kyprolis®), its oral analog ONX 0912 (oprozomib), marizomib (salinosporamide A/NPI-0052), the β5i-specific inhibitors ONX 0914 and PR-924, and the α7-inhibitor SAHQ. Both T-ALL (CEM) cells and MM (8226) cells showed sensitivity to all proteasome inhibitors in the nanomolar range, except for SAHQ and PR-924 which had µM range efficacy. Cells were most sensitive for carfilzomib. Acute leukemia patient cells at diagnosis (chapter 7) were also most sensitive for carfilzomib, followed by bortezomib, ONX 0912 (oprozomib), and ONX 0914. Of all active site proteasome inhibitors evaluated, acute leukemia cells displayed least sensitivity to the β5i-specific inhibitor ONX 0914. This was unexpected since acute leukemia cells express predominantly immunoproteasomes rather than constitutive proteasomes. It was previously established by Parlati et al. that beyond inhibition of the β5i subunit, inhibition of its constitutive counterpart β5 is necessary to elicit an anti-myeloma effect. Subsequently, this notion was underscored in studies described by us (chapter 4) and by Singh et al. for PR-924, showing that this specific immunoproteasome inhibitor also only elicited cell death when drug concentrations were reached that targeted both β5i and β5 subunits. Next to its toxicity, another disadvantage of bortezomib is its binding to red blood cell proteasome and its slow dissociation thereof. In fact, slowly reversible and irreversible inhibitors share binding to proteasomes in red blood cells, vascular endothelium, and well-perfused organs, which may act as a sink for these drugs, thereby impairing distribution to target cells. While a shift can be noted from reversible inhibitors to irreversible inhibitors, the differences in recovery of chymotrypsin-like proteasome activity inhibition after treatment with the slowly reversible inhibitor bortezomib and the irreversible inhibitor carfilzomib are only minor. In addition, also similar recovery times were noted after marizomib, ONX 0912, bortezomib, and CEP-18770 administration, probably due to proficient capacity of cells to synthesize new proteasomes. Ixazomib (MLN9708), which is a rapidly reversible oral proteasome inhibitor with a faster dissociation rate from red blood cells, will hold promise for improved tissue distribution compared with other inhibitors. This indicates that beyond proteasome activity recovery, the binding kinetics and tissue distribution of these agents are at least equally important, which might be especially of importance in extramedullary disease. Since outcomes with single-agent bortezomib were unsatisfactory, combination therapies with conventional chemotherapeutics, HDAC inhibitors, or other proteasome inhibitors - as outlined in chapter 2 – were pursued proving much more promising. The therapeutic benefit of combination therapies involving bortezomib and carfilzomib in MM and lymphoma patients set the stage to exploit next generation proteasome inhibitors for MM as well as leukemia. The observations that mechanisms of action of proteasome inhibitors do not overlap with
other leukemic drugs indicates that proteasome inhibitors can be a valuable extension to current chemotherapy for patients with acute leukemia, especially for drug resistant patients.

LONG-TERM EFFECTS OF PROTEASOME INHIBITORS AND DEVELOPMENT OF RESISTANCE (IN VITRO)

Despite the promising results obtained with bortezomib treatment, resistance to bortezomib may occur, hindering its therapeutic activity\(^1\). To further understand the underlying molecular basis for this, we developed bortezomib-resistant model cell lines of hematologic malignancies, which displayed mechanisms of acquired bortezomib resistance, such as the acquisition of \(PSMB5\) subunit mutations found by our and several other groups\(^11,14-17\) (chapter 3). These mutations form a cluster region in the \(\beta5\) subunit and computational analysis of the effect of the mutations on bortezomib-binding suggested that these mutations compromise bortezomib-binding, as underscored by Suzuki et al. in bortezomib-resistant adenocarcinoma cells\(^1\). It took about a year to obtain an appreciable resistance level to bortezomib in the MM cell line 8226, while the acute leukemia cell lines developed resistance in 15-20 weeks, indicating that acute leukemia cells are more prone to resistance development. In addition, in chapters 4-5 we showed that leukemia cells with acquired resistance to the immunoproteasome inhibitor PR-924 or marizomib (salinosporamide A) share a common molecular mechanism to bortezomib-resistance, i.e. acquisition of point mutations in the constitutive \(\beta5\) subunit, and not in the \(\beta5i\) immunoproteasome subunit. In contrast to bortezomib-resistant CEM cells, which acquired a mutation at resistance to 7 nM bortezomib (i.e. two-fold \(IC_{50}\) concentration), CEM cells resistant to salinosporamide A only developed a mutation at 20 nM (i.e 5-fold \(IC_{50}\) concentration), suggesting that salinosporamide A is more promising in avoiding resistance development. Although in general, cross-resistance of next generation proteasome inhibitors to bortezomib was observed, they retained activity in bortezomib-resistant cells. Also PR-924 resistant cells displayed cross-resistance to carfilzomib and ONX 0914, underscoring the effects of common resistance mechanisms to all proteasome inhibitors. In contrast, Suzuki et al. showed that bortezomib-resistant adenocarcinoma cells harboring \(PSMB5\) mutations retained sensitivity for carfilzomib\(^1\). Altogether, the acquisition of a mutation in the constitutive \(\beta5\) subunit commonly occurs in tumor cell lines resistant to proteasome inhibitors but this will probably not be the only mechanism required for complete resistance, as was indicated by Ri et al\(^1\) who showed that transfecting mutant \(PSMB5\) in a MM cell line partially conferred resistance to bortezomib, but not to the same extent as bortezomib-selected resistant KMS-11/BTZ cells.

As a second resistance mechanism, we (chapters 3-5) and others point to upregulation of \(\beta5\) subunit expression as a primary response mechanism to bortezomib\(^14,15,18-20\), which next may set the stage for acquisition of mutations following prolonged bortezomib exposure. In addition, we found that besides constitutive proteasome subunit expression also the catalytic activity of the constitutive subunit was upregulated in bortezomib-resistant hematologic cells compared to parental cells, whereas immunoproteasome subunit expression and catalytic activity were downregulated. Likewise, bortezomib-resistant adenocarcinoma cells, NSCLC cells, and EBV-transformed B-lymphoblastic JY cells also displayed a marked increase in constitutive subunit expression. Remarkably, however, in contrast to bortezomib-resistant
hematologic cells, these bortezomib-resistant cells were characterized by a concomitant induction of immunoproteasome expression\textsuperscript{11,21,22}. This indicates that bortezomib resistance has a differential impact on constitutive and immunoproteasome composition between leukemic cell-types versus normal lymphoid cells and solid tumor cells. Whether or not this relates to the ability of hematologic cells to assemble of constitutive and immunoproteasome subunits in hybrid forms is not known\textsuperscript{23}.

Tumor cells have the capacity to modulate immunoproteasome function to escape immune surveillance\textsuperscript{24}. This condition may also arise in hematologic tumor cells with acquired resistance to bortezomib due to the acquisition of mutations in the \textit{PSMB5} gene encoding the constitutive β5 subunit. Since its immunoproteasome β5i counterpart does not harbor mutations, downregulation of immunoproteasome in bortezomib-resistant hematologic tumor cell lines may provide a mechanism to escape targeting by bortezomib. We consistently showed that upregulation of β5 protein preceded the acquired mutations. Considering this altered proteasome expression in resistant cells, we tried to overcome resistance by normalizing this disbalance in subunit expression by exposure to IFN-γ. We documented for the first time the impact of IFN-γ on constitutive- and immunoproteasome homeostasis in three bortezomib-resistant tumor cell lines of different hematologic origin (chapter 6). Characteristically, IFN-γ increased the expression of catalytically active immunoproteasome levels in bortezomib-resistant cells with concurrent downregulation of both mutated and unmutated alleles of constitutive β5. This effect facilitated sensitization to bortezomib, and an even more pronounced sensitization to the immunoproteasome inhibitor ONX 0914. This would be fully compatible with the notion that the incorporation of immunoproteasome subunits confers structural changes in the 20S proteasome complex, facilitating accessibility of ONX 0914 to the active sites\textsuperscript{25}. Hence, strategies that would increase immunoproteasome expression merit further exploration as therapeutic modality. Of additional mechanistic support of this concept was that β5i knockdown in THP1 cells abrogated sensitivity to ONX 0914, whereas IFN-γ-induced upregulation of β5i sensitized for ONX 0914. In addition, β5 downregulation in THP1 cells increased sensitivity to bortezomib\textsuperscript{14,26}. Accordingly, knockdown experiments revealed that β5i expression is critically involved in mediating the proteasome inhibitor-sensitizing effects in bortezomib-resistant tumor cells\textsuperscript{27}. The impact of β5i may first be related to proteasome assembly, in which β5i is required for processing and incorporating the β1i and β2i subunits\textsuperscript{28}. Consistently, β5i deficiency delays immunoproteasome assembly\textsuperscript{29}. It will be interesting to further decipher the mechanisms regulating proteasome homeostasis and in particular the equilibrium between the assembly of the immunoproteasome vs. constitutive proteasome in AML and ALL.

A different mechanism to overcome resistance to conventional proteasome inhibitors may come from structural α-subunit inhibitors such as 5AHQ\textsuperscript{7}. The α7-inhibitor 5AHQ was able to completely overcome bortezomib-resistance in the THP1/BTZ500 cell line as well as in bortezomib-resistant NSCLC cells\textsuperscript{21}. However, because of pharmacokinetic limitations, this inhibitor did not reach evaluation in clinical trials and no other α-subunit inhibitor was taken for further development yet. Furthermore, upstream inhibitors of the proteasome such as b-AP15, an inhibitor of deubiquitinating activity was developed and tested\textsuperscript{30}. Other than 5AHQ, b-AP15 did not affect proteolytic 20S activity\textsuperscript{30,31}. The drugs, however, showed significant leukemia regression in an AML mouse model\textsuperscript{10} and was active against MM patient cells, even in those that had relapsed after bortezomib therapy\textsuperscript{32}. Furthermore,
b-AP15 showed synergistic activity with dexamethasone and lenalidomide in an MM cell line. Assessment of the expression levels of the targets of b-AP15 (USP14 and UCHL5) in leukemic cells should reveal its potential for therapeutic interventions.

The immunomodulatory drug lenalidomide, used for MM treatment, was recently identified as an E3 ubiquitin-ligase inhibitor. As shown previously by Chauhan et al., salinosporamide A and lenalidomide displayed synergistic proteasome activity inhibition in MM cell lines, arguing for its involvement in proteasomal degradation. With its recent approval for AML patients, it will be interesting to investigate these combinations in further detail. In addition, MLN 4924 is a more upstream inhibitor of the ubiquitin-proteasome system (UPS). It inhibits the NEDD8-activating enzyme (NAE), a regulator of RING-E3-ligase, involved in regulating the ubiquitinylation rate. Inhibition of NAE leads to turnover of proteins whose ubiquitinylation is mediated by RING-E3-ligases, which occurs for approximately 20% of all proteins. Interestingly, MLN 4924 has shown anti-leukemic activity in AML, both in primary patient cells and mouse models. Since these upstream UPS inhibitors confer a more selective targeting compared to the current proteasome inhibitors, these drugs will be promising for the circumvention of off-target effects and toxicities of proteasome inhibitors.

The original rationale to employ bortezomib in patients with MM and leukemia was based on observations that conventional chemotherapeutics frequently activate the NF-κB pro-survival pathway and that bortezomib would dampen this process by abrogating the proteasomal degradation of IκB, NF-κB’s natural inhibitor. As such, the NF-κB pathway is constitutively active in cells of hematologic cancers. In addition, bortezomib-resistant constitutive NF-κB activity is frequently observed in primary mantle-cell lymphoma samples. Though we showed a significant decline in NF-κB activity in pre-B-ALL patient cells 24h after bortezomib administration compared to pre-treatment samples, this decline in NF-κB activity did not correlate with response to bortezomib, underscoring previous findings by Hideshima et al., who indicated that the cytotoxicity of bortezomib was not associated with NF-κB inhibition. The role of NF-κB inhibition by bortezomib therefore remains controversial and cell type-specific. Furthermore, NF-κB can lead to the upregulation of multidrug efflux transporter MDR1/P-glycoprotein (Pgp/ABCB1) expression, which is a well-studied contributor of bortezomib-resistance. Bortezomib is considered to be a poor substrate for Pgp, in contrast to the epoxyketone-based proteasome inhibitors, which are proficient substrates for Pgp. Nevertheless, Pgp did not play a role in resistance to PR-924 (chapter 4). A relatively new topic of interest is the loss of X-box binding protein (Xbp1) signaling which induced bortezomib-resistance in MM cell lines and patient cells and will be interesting to study further. Other possible mechanisms of resistance to bortezomib in myeloma are caused by bone marrow stromal cells and upregulation of IGF-1 in MM cell lines and primary plasma cells which form new potential treatment targets. Together, multifactorial mechanisms may contribute in conferring resistance to bortezomib in in vitro model systems of leukemic cells (summarized in Figure 1).

**LONG-TERM EFFECTS OF PROTEASOME INHIBITORS AND DEVELOPMENT OF RESISTANCE (IN VIVO)**

Although the leukemia patient samples evaluated in this thesis displayed differential sensitivity to proteasome inhibitors, this was unrelated to the acquisition of mutations in the β5 subunit of the proteasome. It should be noted, however, that most patient samples
General discussion and future perspectives

Figure 1. Overview of molecular mechanisms contributing to acquired resistance to bortezomib in in vitro model systems of hematological malignancies. Resistance mechanism depicted include; (1) upregulation of constitutive proteasome (cP) subunit expression, in particular β5, requiring increased concentrations of proteasome inhibitors (PI) to achieve sufficient inhibition of chymotrypsin-like proteasome catalytic activity, (2) Point mutations in PSMB5 gene introducing amino acid substitutions in critical positions of the PI-binding pocket of the β5 subunit, (3) Down-regulation of immunoproteasome (iP) subunit β5i to escape inhibitory activity of PI, (4) Cellular extrusion of PI facilitated by the drug efflux transporter P-glycoprotein (most relevant for epoxyketone-based proteasome inhibitors), (5) activation of pro-survival pathways, e.g. PI3K/Akt through insulin-like growth factor-1 receptor (IGFR) signaling or NF-κB activation via growth factors and cytokines derived from stromal cells, and (6) the loss of Xbp1 renders cells resistant to bortezomib. * denotes β5 subunit mutation. Modified from 51.

used were obtained prior to treatment and the post-treatment samples were obtained from patients already after their first treatment cycle of 3 weeks. Since all β5 mutations found in human hematologic cell lines were acquired after prolonged bortezomib exposure, rationally mutations will take a longer time period and multiple treatment cycles to develop. From this perspective, it may not be surprising that in the clinical setting, no β5 mutations were (yet) identified in patients treated with bortezomib52–54. It will therefore be of interest to examine whether or not proteasome subunit mutations will emerge during maintenance therapy with proteasome inhibitors, which warrants further evaluation when the efficacy of ixazomib (MLN9708) will be explored as maintenance therapy for MM patients. Intriguingly, PSMB5 mutations in relation to bortezomib resistance were reported as an underlying mechanism of self-resistance to the proteasome inhibitor salinosporamide A in actinobacterium Salinispora tropica55. This observation demonstrates that proteasome β-subunit mutations have an evolutionary ancestor in conferring proteasome inhibitor resistance. Research has also been dedicated on whether or not polymorphic variations of proteasome subunit genes
Gene resequencing identified polymorphism Arg24Cys in PSMB5, which was more prevalent in patients with MM treated with bortezomib compared to healthy individuals. However, this and other polymorphisms did not influence proteasome activity or sensitivity to proteasome inhibitors. Lichter et al. also did not find an association between PSMB5 polymorphisms and response to bortezomib or dexamethasone in relapsed MM patients treated in the APEX study. These data indicate that these PSMB5 polymorphisms do not arise from selective pressure during bortezomib-treatment.

In contrast to mutations in PSMB5, mutations in PSMB8 (encoding β5i) were implicated in the development of autoimmune diseases, and a Phe50Ile mutation in the propeptide of PSMB8 was found in bortezomib-resistant adenocarcinoma cells. However, also no alterations were found in PSMB8 in the cell lines or patient samples in this thesis. In MM patients, polymorphism Q49K (Glu49Lys) was commonly found, however, more in pre-treatment samples than in samples after bortezomib treatment and was not linked to bortezomib-response. Nonetheless, a patient with CANDLE syndrome displayed a Q49K variation in PSMB8. LMP7-K/K homozygous cell lines showed reduced PSMB8 mRNA levels due to reduced transcript stability and in addition a lower increase in HLA class I expression after IFN-γ stimulation compared to LMP7-Q/Q cell lines. This polymorphism is located in the presequence, and thus absent in the mature protein. It can however impact assembly of the immunoproteasome and is therefore interesting to study further in larger datasets of (bortezomib-treated) leukemia patients.

To determine if alterations in proteasome composition observed in vitro will also be clinically relevant, we evaluated proteasome subunit expression in ALL and AML patient cells at initial diagnosis ex vivo (chapter 7) and at diagnosis of relapse in vivo (chapter 8). Consistently, we found that total proteasome levels did not differ between ALL and AML cells; however, ALL cells had significantly lower expression of constitutive subunits and higher β1i expression than AML cells. Accordingly, ALL patient cells at diagnosis were ex vivo significantly more sensitive to proteasome inhibitors compared to AML cells. In addition, patients with a higher immune/constitutive proteasome ratio responded better to bortezomib-containing treatment in vivo compared to patients with a lower ratio. The notion that a lower immunoproteasome subunit expression and higher constitutive subunit expression can be an accountable factor in resistance to bortezomib was underscored by Lü et al. showing that a MM patient displayed higher PSMB5 mRNA expression after bortezomib treatment compared to before treatment. Also, Leung-Hagesteijn et al. showed decreased immunoproteasome expression in bortezomib-resistant MM patients compared to bortezomib-sensitive patients. Based on these considerations, strategies that may increase immunoproteasome levels may merit further exploration for therapeutic intervention. Unfortunately, the clinical use of IFN-γ and IFN-α has not been very effective to date and often comes with side effects. Alternatively, lipid A, the endotoxic moiety of gram-negative bacterial lipopolysaccharide incorporated into liposomes was shown to induce secretion of IFN-γ resulting in a shift from constitutive proteasomes to immunoproteasomes and will be interesting to explore as adjuvant treatment to proteasome inhibitors in bortezomib-resistant patients.
FUTURE PERSPECTIVES

Although major improvements have been achieved in the treatment of pediatric and adult leukemia patients, the prevention of relapse and further improvement of survival of relapsed patients still leaves many (pre-)clinically directed challenges. In the upcoming years, we anticipate further attempts at designing fine-tuned treatment protocols involving a proteasome inhibitor combined with classical and novel compounds with proven efficacy in leukemia treatment, such as HDAC inhibitors and glucocorticoids. To minimize bortezomib-induced peripheral neuropathy, it will be possible to switch to the orally available next-generation proteasome inhibitors, for example, ixazomib (MLN9708) or oprozomib. Ixazomib has shown similar efficacy as bortezomib in MM patients, but because of its oral administration, optimized kinetics and a low incidence of peripheral neuropathy, ixazomib merits testing in the clinical leukemic setting. Importantly, the upcoming study of ixazomib as maintenance therapy of MM patients will shed light on the efficacy of prolonged proteasome inhibitor treatment. Add-on studies to clinical trials should further expand our knowledge on the pharmacokinetic properties of each type of proteasome inhibitor (reversible and irreversible) and to identify parameters of response and/or onset of drug resistance and unraveling the molecular basis thereof. In addition, possible resistance mechanisms such as Pgp-mediated drug extrusion and Xbp1 expression should be monitored for involvement in response to (epoxyketone-based) proteasome inhibitors. Furthermore, there is an absolute need for randomized clinical trials to be able to compare the added value of proteasome inhibitors to the conventional treatment of leukemia. Because of the increasing interest into the role of the immunoproteasome, knowledge of the dynamics of constitutive- and immunoproteasome expression, hybrid proteasomes, and their differential regulation in leukemic cell subtypes deserves further exploration to build better prediction models for therapy response. In this context, our data indicate that ratios of immunoproteasome and constitutive proteasome subunit expression may serve as one of these parameters for assessment of proteasome inhibitor sensitivity in leukemic patients. To establish whether the immuno/constitutive proteasome subunit ratio indeed represents a contributing factor in proteasome inhibitor response deserves further investigations in larger size patient cohorts.

In summary, the results described in this thesis provide a sound rationale for incorporating proteasome inhibitors in the treatment of (relapsed) acute leukemia. Optimal use should come from combination therapies with other conventional chemotherapeutics to diminish drug resistance. Examinations of prevailing mechanisms of drug resistance in clinical specimen of therapy-refractory patients warrant further investigation but results may depend on cell types, type of proteasome inhibitor and duration of drug administration.
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