Molecular mechanisms of bortezomib resistance in acute leukemia

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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.