Proteasome inhibitors in acute leukemia

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SUMMARY - PROTEASOME INHIBITORS IN ACUTE LEUKEMIA

Acute leukemia comprises about 30% of all pediatric cancers. In the Netherlands, each year about 120 children are diagnosed with the most common type, acute lymphoblastic leukemia (ALL). Survival of pediatric patients with leukemia has greatly improved in recent decades. However, 20-30% of patients relapse and outcome after relapse remains poor, with remission rates as low as 10–40% for poor prognostic subtypes. Thus, there is an ongoing need for improvement in therapeutic options for these patients. Based on the clinical activity in adult hematologic malignancies, such as multiple myeloma (MM) and mantle cell lymphoma, proteasome inhibitors, particularly bortezomib, are now being explored in the clinical setting in childhood acute leukemias. Proteasomes are responsible for the degradation of the majority of intracellular proteins in all cells. Misfolded, damaged, or even newly synthesized proteins will be marked for degradation after ubiquitin-conjugation. Upon protein degradation, short peptides are generated that can either be presented on major histocompatibility complex (MHC) class I molecules on the cell surface to initiate an immune response, or further hydrolyzed by aminopeptidases to amino acids reutilized for protein synthesis.

In this thesis we focused on the anti-leukemic effects of bortezomib and next generation proteasome inhibitors against human leukemic cell line models and *ex vivo* clinical samples from pediatric patients. Because emergence of acquired resistance is often observed during bortezomib therapy of MM patients, and thus a potential limiting factor of the efficacy of proteasome inhibitor treatment, we also aimed to further decipher the molecular basis of acquired resistance to proteasome inhibitors in leukemia cells. Based on these studies, we set out to define parameters that could help to identify patients who may benefit from bortezomib-containing therapy and/or examine strategies to overcome resistance to bortezomib.

Chapter 2 covers a review on proteasome inhibitors in acute leukemia. The different classes of proteasome inhibitors with their clinical (dis)advantages are described. Furthermore, the current status of proteasome inhibitors (for pre-clinical and clinical studies) in the treatment of acute leukemia is outlined, which builds on the results obtained with bortezomib in MM therapy. Furthermore, an overview is presented on the combinations of proteasome inhibitor therapy with conventional chemotherapeutics, other proteasome inhibitors, or histone deacetylase inhibitors. Shifting from intravenous to orally available proteasome inhibitors, next to the decline in toxicities with next generation proteasome inhibitors, should also add to improved clinical benefit. Optimal management of leukemia should come from further fine-tuning combination therapies with other conventional chemotherapeutic agents.

Despite the promising results obtained with bortezomib treatment, chemoresistance to bortezomib may occur that could hinder its pharmacologic activity. To unravel potential resistance mechanisms, we developed bortezomib-resistant models of cell lines of hematologic malignancies. These displayed mechanisms of acquired bortezomib-resistance such as upregulation of constitutive proteasomal catalytic subunits as well as acquisition of β5 subunit mutations (**chapter 3**). We showed that these bortezomib-resistant hematologic tumor cells display a cluster of mutations in the S1 bortezomib-binding pocket of *PSMB5*. Based on computational modeling we concluded that these mutations lead to impaired

bortezomib binding to the mutant β5 subunit of the proteasome, pointing out the underlying basis for bortezomib resistance in leukemia cells. Furthermore, the antileukemic effect of a series of next generation proteasome inhibitors was evaluated in bortezomib-resistant cell lines. Though to some extent cross-resistance to bortezomib was noted, bortezomib-resistant hematologic cell lines displayed sensitivity to the epoxyketone-based proteasome inhibitors carfilzomib, ONX 0912, and ONX 0914. Except for the α7 subunit inhibitor 5AHQ, which showed similar sensitivity in bortezomib-resistant and parental cells.

Emergence of resistance to bortezomib and its common side effects, e.g. peripheral neuropathy, has initiated the development of next generation proteasome inhibitors with irreversible vs. reversible proteasome binding properties, better pharmacokinetic properties and/or an optimized therapeutic window. In **Chapters 4-5**, *in vitro* studies are described, examining the antileukemic effects of two next generation proteasome inhibitors against leukemia cell lines, along with exploring potential mechanisms of resistance to these drugs following long term exposure to leukemia cells. Selective inhibition of the immunoproteasome gained considerable attention given their potential activity against immune diseases and inflammatory disorders. **Chapter 4** shows the antileukemic effect of β5i-specific inhibitor PR-924 and the mechanisms of acquired resistance to PR-924 in hematologic tumor cells. PR-924 displayed antileukemic activity, even in bortezomib-resistant cells, but relies on the complementary inhibition of both β5i and constitutive β5 subunits for exerting its antileukemic effect. Upon acquired resistance of CCRF-CEM (T-ALL) cells to PR-924, cells acquired a Met45Ile mutation in the *PSMB5* gene, next to upregulation of constitutive proteasome subunit expression and downregulation of immunoproteasome subunit expression. This underscores that the β5 subunit is a key determinant in mediating drug resistance to proteasome inhibitors, even for primarily immunoproteasome-targeted inhibitors.

Chapter 5 shows the antileukemic effect of the proteasome inhibitor salinosporamide A (Marizomib, NPI-0052), which is produced by marine actinobacterium Salinispora tropica, and reveals the mechanisms of acquired resistance to this drug in CCRF-CEM (T-ALL) cells. In contrast to other proteasome inhibitors, salinosporamide A inhibits all three catalytic proteasome activities. Salinosporamide A displayed significant antileukemic activity in low nanomolar concentrations, and was synergistic with bortezomib in bortezomib-resistant cells. CEM cells displaying 5-fold acquired resistance to salinosporamide A showed cross-resistance to bortezomib. Intriguingly, mutations in salinosporamide A-resistant leukemic cells were shared with the Salinispora tropica actinobacterium itself, pointing to an evolutionary-conserved resistance mechanism.

Thus, leukemia cells with acquired resistance to bortezomib, PR-924, or salinosporamide A have common overlapping molecular mechanism of resistance, i.e. acquisition of point mutations in the constitutive β5 subunit. In addition, catalytic proteasome constitutive subunit activities and subunit expression were upregulated compared to parental cells, whereas immunosubunit expression and catalytic activity were mostly downregulated.

Because of the altered proteasome assembly of bortezomib-resistant tumor cells, the impact of interferon-γ on constitutive- and immunoproteasome homeostasis was investigated in three bortezomib-resistant tumor cell lines of different hematologic

origin (**chapter 6**). Bortezomib-resistant cells that displayed a downregulation of immunoproteasome expression and an upregulation of constitutive proteasome expression compared to parental cells were exposed to interferon-γ. Interferon-γ markedly increased the expression of catalytically active immunoproteasome expression in bortezomib-resistant cells with concurrent downregulation of both mutated and unmutated alleles of constitutive β5. These features resulted in the sensitization of bortezomib-resistant cells for bortezomib, but most prominently for the immunoproteasome inhibitor ONX 0914. This sensitizing effect was abrogated by siRNA silencing of β5i but not by β1i silencing, demonstrating that the β5i subunit is a major player in bortezomib-resistance.

To verify whether or not these *in vitro* determined parameters also hold clinical relevance, we determined proteasome subunit expression *ex vivo* in ALL and AML patient cells obtained at initial diagnosis (**chapter 7**). We showed that proteasome composition of leukemic cells is predominantly comprised of immunoproteasomes. In addition, immunoproteasome subunits are expressed at relatively higher levels in ALL cells compared to AML cells, whereas total proteasome levels did not differ between these two leukemia cell types. *Ex vivo* sensitivity to proteasome inhibitors bortezomib, carfilzomib, ONX 0912 (oprozomib), ONX 0914, 5AHQ and the glucocorticoid dexamethasone showed that ALL cells were significantly more sensitive to all these drugs compared to AML cells. Because in pediatric ALL, a good response to glucocorticoids is a favorable prognostic factor for survival, it was of interest to note that the pharmacological efficacy of bortezomib could be further enhanced in combination with dexamethasone, eliciting additive or synergistic effects. After classification into subgroups, even the prognostically unfavorable subgroups pro-B ALL and T-ALL were equally sensitive to bortezomib as pre-B/common ALL samples. Correlations of proteasome subunit expression and sensitivity to proteasome inhibition revealed that ratios of immunoproteasome to constitutive proteasome subunits significantly correlated with response to proteasome inhibitors in pediatric ALL and AML cells.

In **chapter 8**, we investigated whether ratios of immunoproteasome to constitutive proteasome protein correlate with clinical outcome in first relapsed and refractory pediatric acute leukemia. Patients were enrolled in two Children's Oncology Group (COG) phase 2 clinical trials of bortezomib with reinduction chemotherapy for pediatric acute ALL (COG-AALL07P1) and pediatric AML (COG-AAML07P1). Protein expression of proteasome subunits obtained prior to bortezomib-containing reinduction therapy showed that ratios of both β5i/β5 and β1i/β1 subunit expression were significantly higher in ALL cells than in AML cells. Upon stratification of patients by attainment of complete remission (CR) following induction, ratios of both β5i/β5 and β1i/β1 were significantly higher in patients that attained a CR compared to patients who did not. In addition to protein expression ratios, increased ratios of pre-treatment proteasome subunit-specific catalytic activity of β5i/β5 were observed in patients who reached CR compared to those that did not. Together, these results suggest that higher ratios of immuno/constitutive proteasome in pretreatment acute leukemia can serve as a novel putative predictor for the clinical response to bortezomib-containing treatment.

Although the patient samples evaluated in this thesis displayed differential sensitivity to proteasome inhibitors, this did not coincide with mutations in the β5 subunit of the proteasome. Most patient samples used in this thesis were obtained prior to treatment and

the post-treatment samples were obtained after the first treatment cycle of 3 weeks. Since all β5 mutations found in human cell lines were acquired after prolonged bortezomib exposure, rationally mutations will take longer to develop. Rather, these and other studies point to upregulation of β5 subunit expression as a primary response mechanism to bortezomib, which may set a stage for acquisition of mutations following prolonged bortezomib exposure.

In conclusion, bortezomib and next generation proteasome inhibitors are excellent candidates for implementation in the treatment of childhood acute leukemias. For orally available next generation proteasome inhibitors, the findings described here may hold promise in the future treatment of pediatric leukemia by avoiding toxicity of bortezomib, circumvention of bortezomib resistance and further assessment of their synergistic effect when combined with other drugs including glucocorticoids. Regarding mechanisms of bortezomib-resistance, results described in this thesis imply that leukemic patients harboring higher immunoproteasome expression and (concomitant) lower constitutive proteasome expression before bortezomib treatment will respond better to bortezomib-containing treatment than patients with higher constitutive proteasome expression. To establish whether the immuno/constitutive proteasome ratio represents an additional contributing factor in proteasome inhibitor response deserves further investigations in larger size patient cohorts.

KEY POINTS

1. Based on pre-clinical evaluations, bortezomib combined to conventional chemotherapeutics and next generation proteasome inhibitors appear promising treatment modalities for (relapsed) pediatric acute leukemia.

2. Step-wise increasing concentrations of bortezomib in cells of hematologic origin provoke acquired resistance by upregulation of constitutive proteasome subunits and come with induction of point mutations in the *PSMB5* gene, leading to impairment of bortezomib binding.

3. Immunoproteasome inhibitor PR-924 specifically inhibits the β5i subunit, but only elicits an antileukemic effect when both immunoproteasome subunit β5i and constitutive subunit β5 are targeted.

4. Leukemia cells display sensitivity to the naturally produced proteasome inhibitor salinosporamide A (Marizomib, NPI-0052) and salinosporamide A shows synergy with dexamethasone in bortezomib-resistant cells, thus representing a potential treatment option for pediatric leukemia.

5. Upon acquired resistance to next generation proteasome inhibitors, even an immunoproteasome inhibitor, a mutation in the *PSMB5* gene is provoked, underscoring the importance of the β5 subunit as a key determinant in mediating drug resistance to proteasome inhibitors.

6. Similar mutations in salinosporamide A-resistant *in vitro* derived ALL cells as well as in the *Salinispora tropica* actinobacterium itself, point to an evolutionary-conserved resistance mechanism.

7. Higher ratios of immuno/constitutive proteasome levels in pretreatment pediatric acute leukemia cells at diagnosis and at relapse can serve as a novel putative predictor for the clinical response to bortezomib-containing treatment.