Summary

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Proteasomes and aminopeptidases are responsible for the degradation of intracellular proteins that are misfolded, damaged or at the end of their functional life span. These proteins can then be recycled for new protein synthesis. Recognition by the proteasome is mediated by linking single or multiple ubiquitin moieties. Aminopeptidases operate downstream of the proteasome by trimming larger peptides into smaller peptides for antigen presentation on MHC I molecules, or facilitate full peptides hydrolysis to amino acids reutilized for proteins synthesis. The protein degradation pathway (Chapter 1) is important both in normal homeostasis and in disease settings, such as autoimmune diseases and cancer, and its components thus make up attractive targets for therapeutic intervention.

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease which affects the synovial joints of approximately 1% of the population of developed regions. The cause of this disease is still unclear, but likely involves a combination of genetic factors and/or environmental triggers. Chronic inflammation of the synovial joints is accompanied by hyperplasia of synovial tissue, autoantibody production, and cartilage and bone destruction, of which the underlying cause lies in immune regulatory factors such as the loss of tolerance. How these processes are linked to a localized onset of inflammation in the joint is still unclear but it involves immune effector cells including macrophages and osteoclasts, myeloid and plasmacytoid dendritic cells (DCs), B cells and T cells. To date, only 50% of RA patients benefit from the current treatment strategies consisting of the use of combinations of conventional Disease Modifying Anti Rheumatic Drugs (DMARDs) with biological agents. As a result, there is a continuous quest to identify and develop more efficacious, less toxic and less expensive drugs (Chapter 1).

Proteasomes are known to process key proteins involved in the functioning of immune effector cells that participate in the pathology of RA as well as other autoimmune diseases, including systemic lupus erythematosus (SLE), Sjögren’s Syndrome (SS) and sclerodema. One example is the degradation of IκB which normally activates the NF-κB pathway promoting the transcription of pro-inflammatory cytokines which drive inflammation. Other vital processes that
are controlled by proteasomes include cell cycle regulation, apoptosis and MHC-mediated antigen presentation. Interfering with these processes using therapeutic agents that block the catalytic action of proteasomes offers an alternative way to combat (chronic) inflammation. Proteasome inhibitors (PIs) may therefore hold potential as anti-inflammatory agents.

While PIs are still in the exploratory phase as anti-inflammatory drugs, their application in cancer chemotherapy has grown rapidly. In fact, several PIs have been discovered and developed in the last 10 years (Chapter 2) which vary in class (the way they bind – reversibly or irreversibly - to proteasomes), target (the types of proteasomes they bind to) and route of administration (if already clinically applicable). Bortezomib (BTZ) is the first prototypical PI that was registered for treatment of therapy-refractory multiple myeloma patients and next-generation PIs, including carfilzomib (CFZ), ONX 0912 and ONX 0914, have been designed and are currently being tested in several hematological malignancies.

Toxicity profiles that were originally reported in BTZ-containing anticancer therapies could be a limiting factor for its long term use as an anti-inflammatory agent, but nowadays toxicities are better manageable and may be less harsh for the next generation PIs.

At first glance, it might seem remarkable to explore drugs with similar mechanisms of action for the treatment of two diseases with different backgrounds, i.e. autoimmune diseases and cancer. The manifestation of chronic inflammation in autoimmune diseases is often due to a hyper-active immune system vs. an immune system that has often failed to recognize and eliminate malignant cells in cancer. Immune-modulation-based treatment objectives in autoimmunity aim to dampen inflammation whereas in a cancer the main objective is to boost the immune system and eliminate malignant cells. Notwithstanding these facts, there are overlapping drug targets (e.g. NF-κB activation) and efficacy issues (resistance development) which are important in both settings. Additionally, many lessons can be learned from the treatment of autoimmunity when examining cancer immunotherapy (and vice versa), which is actually aimed at the induction of a kind of autoimmunity directed against
tumor-related auto-antigens.

In this thesis, we set out to address two main goals:

1. To explore the short and long term effects of inhibiting the protein degradation pathways in immune effector cells implicated in the pathophysiology of autoimmune diseases and cancer progression.
2. To define molecular mechanisms that may be responsible for loss of efficacy to proteasome and aminopeptidase inhibitors through induction of acquired resistance in various immune effector cells.

Studies on experimental drugs interfering with the protein degradation pathway via PIs are presented in Chapters 2 to 6, those for aminopeptidase inhibitors are discussed in Chapters 7 and 8.

Chapter 1 provides a brief overview of the pathophysiology, current treatment modalities and the window of opportunity for innovative therapeutics in RA, and proposes the processes of protein synthesis and degradation as viable targets for therapeutic intervention by inhibitors of the proteasome and aminopeptidases.

The current state of PIs in as experimental anti-inflammatory drugs is discussed in Chapter 2 by reviewing information on the diversity and relevance of proteasome subtypes in immune-competent cells in relation to autoimmune diseases. In this review, an up-to-date overview of the several classes of clinically available and new generation rationally-designed experimental PIs is given. Specifically, pre-clinical data related to the impact of BTZ and next-generation PIs on functional properties of immune-effector cells including T cells, B cells, dendritic cells, macrophages and osteoclasts are presented. Also reported mechanisms of PI resistance are highlighted, including: 1) altered proteasome subunit expression levels, 2) mutations located in the BTZ binding pocket, 3) MDR1 (Pgp) –mediated drug efflux, 4) increased levels of heat shock proteins, and 5) increased activation of Insulin-like Growth Factor-1 (IGF-1) receptor transducing a pro-survival signal. Lastly, this chapter discusses the
current (pre-)clinical status of PIs and their future perspectives in serving a role as anti-inflammatory (and anti-arthritic) agents in a clinical setting.

Dendritic cells, the most powerful professional antigen presenting cells (APCs), also control other players and processes of the immune system through the release of regulatory or stimulatory cytokines, depending on their developmental stage and activation state. In Chapter 3 we assessed the effects of long-term BTZ exposure in a cell line model system of human dendritic cell development (MUTZ-3). Investigations were focused on the impact on DC differentiation and maturation and the assessment of alterations in signalling pathways implicated in DC development and function and revealed enhanced DC development and functionality. These observations imply that the impact of long-term BTZ exposure on DCs would be beneficial for primary application of this drug in cancer, e.g. in combination with immunotherapeutic DC-based strategies.

Since B cells play a pivotal role in the pathogenesis of various autoimmune diseases, they represent attractive targets for therapeutic intervention. In Chapter 4 we examined the effects of long-term BTZ exposure to the human B lymphoblastoid cell line JY. The molecular basis of acquired resistance to BTZ in these cells included a BTZ-induced single point mutation in the PSMB5 gene (encoding the constitutive β5 proteasome subunit) as well as a selective over-expression of mutant PSMB5 protein. In exploring strategies to overcome BTZ resistance, new generation epoxyketone-based irreversible PIs revealed the capacity to do so. This may be due to the fact that in JY/BTZ cells, unlike other BTZ-resistant cells, levels of immunoproteasomes were not down-regulated, and could thus be targeted by the epoxyketone-based PIs. Interestingly, BTZ resistance in JY cells was also accompanied by increased expression of CD20, as a result of impaired breakdown, which set the stage for enhanced complement-dependent cytotoxicity (CDC) induced by anti-CD20/rituximab.

In Chapter 5 we investigated whether next-generation irreversibly binding PIs, including CFZ, ONX 0912 and ONX 0914, might overcome two established BTZ resistance mechanisms in model systems of immune competent cells, i.e. point mutations in the PSMB5 gene or cellular extrusion by multidrug resistance
efflux transporters of the family of ATP-Binding cassette transporters. Evidence was provided that only ABCB1 (Pgp/MDR1) harbored the ability to extrude these drugs and conferred drug resistance in a cell line model with ABCB1(Pgp/MDR1)-overexpression. However, in an ex vivo setting with peripheral blood mononuclear cells (PMBCs) from healthy controls and RA patients, basal ABCB1(Pgp/MDR1) activities were rather modest and still allowed for the retention of proficient proteasome inhibitory capacity by CFZ, ONX 0912 and ONX 0914.

**Chapter 6** reports on short-term effects of proteasome inhibition by BTZ and by the next-generation immunoproteasome inhibitor ONX914 on differentiation and maturation of inflammatory DCs generated from monocytes in the presence of IFN-α, which have previously been implicated in several autoimmune diseases. From this study we concluded that specific immunoproteasome inhibition by ONX 0914 is equally effective in impairing inflammatory IL4- and IFN-DC development and functionality as the clinically registered PI, which, through combined inhibition of both constitutive and immunoproteasome activity, may induce more unwanted side effects.

Aminopeptidases operate downstream of the proteasome in the cellular process of protein/peptide degradation. **Chapter 7** covers an overview of various types of aminopeptidases and their roles in immune-effector cells and malignant cells. The positioning of aminopeptidase inhibitors in cancer and autoimmune disease therapy schedules is discussed.

In **Chapter 8** we investigated the molecular mechanism underlying acquired resistance to the aminopeptidase inhibitor prodrug CHR-2863 in human U937 myelomonocytic cells. A multifactorial mechanism was identified, including (a) impaired prodrug conversion associated with down-regulation of the converting enzyme carboxylesterase 1 (CES1), (b) intracellular sequestration of CHR2863, presumably in lipid droplets, and (c) activation of the Akt/mTOR pro-survival pathway. The latter coincided with a striking gain of sensitivity to the mTOR inhibitor rapamycin. These results indicate that initial inhibition of mTOR activity by aminopeptidase
inhibition-associated amino acid depletion is overcome during development of resistance to CHR2863. What mechanism is responsible for the observed activation of mTOR in CHR2863-resistant cells remains to be established.

In conclusion, (next-generation) proteasome and aminopeptidase inhibitors are excellent candidates for therapeutic immune intervention in both cancer and chronic inflammation. A growing insight into their long-term effects and resistance mechanisms, as provided by the studies described in this thesis, will form the basis for their rational implementation in clinical settings.
1. Pre-clinical evaluation of BTZ and next-generation PIs for treatment of autoimmune diseases is generally promising; further clinical evaluation is eagerly awaited.

2. Chronic exposure to BTZ enhances development and maturation of Langerhans cells from dendritic cell (DC) progenitors, thus arguing for applying BTZ in combination with DC-based cancer immunotherapy modalities.

3. Long-term exposure to BTZ provokes acquired resistance in human B lymphoblastoid JY cells by induction of a point mutation in the PSMB5 gene encoding the highly conserved BTZ-binding pocket of the β5 subunit protein.

4. BTZ-resistant JY cells featured impaired breakdown of CD20, which sensitized these cells to anti-CD20/rituximab-mediated complement dependent cytotoxicity, thus providing a rationale to combine Rituximab and BTZ, to overcome BTZ resistance on the one hand as well as down-regulation of CD20 as a resistance mechanism to Rituximab on the other.

5. Epoxyketone-based next-generation PIs are prone to be extruded via the drug efflux transporter Pgp (MDR1) which thus confers resistance. In an *ex vivo* setting, a low basal Pgp activity in PBMCs of healthy donors and RA patients only modestly influenced the proteasome inhibitory potential of these drugs.
6. Specific immunoproteasome inhibition by the next-generation PI ONX 0914 is equally effective in impairing inflammatory IL4- and IFN-DC development and functionality as inhibition of both constitutive and immunoproteasomes by clinically active BTZ, with potentially less clinical side effects.

7. Aminopeptidases emerge as attractive candidates for therapeutic intervention and rationally designed novel aminopeptidase inhibitors merit further (pre) clinical evaluation.

8. Chronic exposure to the aminopeptidase inhibitor prodrug CHR2863, a close structural analogue of the clinically active aminopeptidase inhibitor Tosedostat, results in the onset of acquired resistance in human myelomonocytic U937 cells.

9. The molecular basis of acquired resistance to CHR2863 is multifactorial, including; a) down-regulation of CES1 expression (enzyme responsible for its conversion), b) impaired prodrug conversion, c) intracellular sequestration of the (hydrophobic) prodrug (presumably in lipid droplets), and d) activation of the Akt/mTOR pro-survival pathway.