The use of activity based protein profiling to study proteasome biology

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Chapter 1: General Introduction

The ubiquitin-proteasome system

Protein degradation is essential for cellular homeostasis and thus for cell survival. Having a regulated protein degradation machinery is crucial to protect functional proteins from degradation, to control proteins half-life or to degrade misfolded or damaged proteins which can be harmful to the cells. The ubiquitin-proteasome system (UPS) is the main degradation pathway in eukaryotes [1, 2]. The UPS marks the proteins for degradation with a poly-ubiquitin chain by means of three different enzyme families which work in cascade [3] to first identify the substrate and then attach to it a poly-ubiquitin chain (figure 1). Ubiquitin itself is a small protein (76 amino acids) that is mostly used as a post-translational modification (PTM) as effected in a cascade of reactions executed by three different types of enzyme families known as E1, E2 and E3 (figure 1). In this process, an isopeptidic linkage is produced starting from lysine side chain amines and the C-terminal carboxylate of ubiquitin. Ubiquitination of proteins can regulate the substrate cellular localization, control its degradation and plays a role in protein-protein interactions. All these cellular processes are regulated by a variety of ubiquitin modifications. Protein substrates can be modified with a single ubiquitin molecule or with a poly-ubiquitin chain. Ubiquitin has seven different lysine residues through which they can be linked to each other to build a poly-ubiquitin chain. These chains can be linear, branched or mixed with other ubiquitin-like molecules. The best-characterized poly-ubiquitin chains are so far the lysine 63- and lysine 48-linked chains while for the other types little is known. Lysine 48 (K48)-linked poly-ubiquitinated proteins are directed towards the proteasome where they are processed into small oligopeptide fragments. The majority of these peptides are further recycled into single amino acids by different peptidases, but a small fraction (estimated to be about 1%) of the peptides generated by the proteasome and partially processed by downstream aminopeptidases are presented on MHC-class I molecules for presentation to the immune system. CD8-positive cytotoxic T cells have developed to recognize peptide-loaded MHC class I molecules and to discriminate between self-peptides and foreign peptides. In this fashion, the UBP plays an important role in immunity and assists in reporting on, for instance, viral infections.

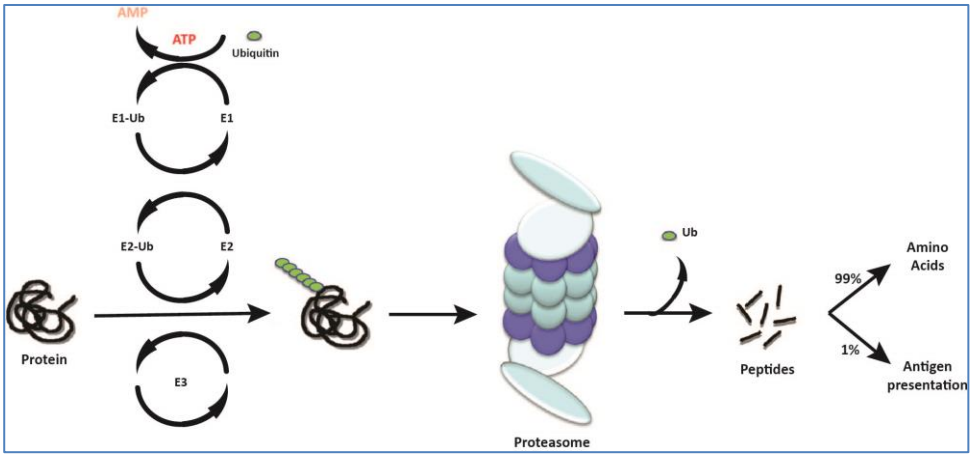

Figure 1. *Schematic representation of the UPS. Ubiquitin is transferred from an E1 ubiquitin transferase to an E2 transferase. This E2-Ub complex binds and transfers the ubiquitin to a protein substrate, which is bound to an E3 enzyme. This last step is repeated (not necessarily by the same pair of enzymes) to build a poly-ubiquitin chain on the substrate, which targets the protein for proteasome degradation. Proteasomes degrade proteins into smaller peptide fragments while the ubiquitin moieties are released and recycled. The generated peptides are further degraded into single amino acids by aminopeptidases. About 1% of the peptides are loaded onto MHC-class I molecules for antigen presentation on the cellular membrane.*

The proteasome is a large protein complex of around 2.5 MDa. It consists of a barrel-shaped core particle, termed 20S, and a small variety of regulatory particles (RP) of which the most common is the 19S RP [4]. The 19S RP binds to one and potentially both sides of the 20S, and triggers an opening to the inside where the catalytic sites are situated. The ubiquitinated substrates are recognized by the 19S RP, which unfold and translocate them into the 20S inner chamber for degradation. The protein will be cleaved into small peptides, which vary between 3 and 15 amino acids in length. The 19S subunit Rpn11 shows deubiquitylating (DUB) activity, which cleaves the bond between the substrate and the poly-ubiquitin chain; this chain will be recycled into single ubiquitin molecules by other DUBs [5]. The 20S proteasome consist of 14 pairs of alpha and beta subunits, which are stacked in rings, being the two alpha rings (each of 7 subunits) on the outer site of the barrel with the two beta rings on the inside. In prokaryotes all seven beta subunits have a catalytic activity which does not differ between subunits, but in eukaryotes only three of the seven beta subunits remain catalytically active, namely β1, β2 and β5. These subunits show differences in their substrate specificity, with β1 cutting preferably the C-terminal of acidic amino acids, β2 after basic ones and β5 rather after bulky or uncharged amino acids [4]. In organisms that have evolved an immune system, the UPS has increased its capability of generating different peptides from a single protein by expressing

immunoproteasomes 20S particles, where the active subunits of the constitutively expressed proteasome (constitutive proteasome, active subunits termed β1c, β2c and β5c) have been replaced by their immunoproteasomes counterparts, β1i, β2i and β5i. These subunits have slightly different cleavage pattern compared to their constitutive counterparts, which has increased the rate of generating peptides suitable for antigen presentation [6, 7]. Having 6 different subunits has expanded the possible pool of proteasomes, since all subunit combinations can be expected, giving rise to hybrids proteasomes in which both immunoproteasome and constitutive proteasome subunits are assembled into the same 20S particle.

Proteasome inhibitors and multiple myeloma

It was thought that disruption of the proteasome was not an option in drug development due to its major role in cellular protein homeostasis. But the discovery of epoxomicin, a broad-spectrum proteasome inhibitor (PI) synthetized by bacteria to fight against fungi infections, which caused cellular apoptosis and the posterior evidence that the UPS regulates cell cycle progression and NFkB signaling, boosted the idea of the UPS blockade as a suitable antineoplastic strategy [8, 9]. Since then major efforts have been made to contribute to this hypothesis, and today two proteasome inhibitors (bortezomib and carfilzomib) have been approved by the FDA for the treatment of mantle cell lymphoma and specifically multiple myeloma (MM) [10, 11]. Currently PIs are being tested in clinical trials alone or in combination with other drugs against a variety of human diseases including breast cancer, arteriosclerosis and Alzheimer's disease.

In the case of MM patients, PIs have evolved from last resort therapy to being the principal treatment. Its phenotype may explain why especially this specific type of cancer is sensitive against proteasome inhibition. MM is a hematopoietic cancer affecting mainly plasma cells, which are fully differentiated B-cells responsible for antibody production. MM plasma cells have a high protein synthesis rate due to the large amount of a single class of antibodies generated for secretion. This high synthesis rate is coupled to a strict quality control check, where misfolded proteins need to be quickly degraded to avoid accumulation or aggregation of misfolded or damaged proteins, which can be detrimental to the cell survival. The proteasome is one of the main players in this quality control system. This difference in protein synthesis rate opens a therapeutic window for the treatment of MM with proteasome inhibitors. The success in extending patients lifespan is clear but PIs are not a cure, and patients always relapse after a certain amount of years. An acquired resistance against proteasome inhibitors is the main drawback in finding an

effective cure. The mechanisms behind the development of adaption are not completely understood [4, 12, 13]. Some studies with PI resistant cell lines suggest that point mutations in the pocket of the β5 subunit, which is the main target of bortezomib and carfilzomib, impede the proper binding of the inhibitors within the active site pocket destabilizing the interaction [14]. Although these cell lines mimic the resistance found in patients, no mutations in the β5 subunit have been found in patients with relapse or refractory myeloma [15, 16]. This suggests that different mechanisms may drive the acquisition of resistance. Most of the secreted proteins, such as immunoglobulin, are synthetized in the endoplasmic reticulum (ER). Misfolded or damaged proteins located in the ER are degraded through the ER associated degradation (ERAD) pathway in which the proteasome is the main protease in charge of their degradation [17]. If the ERAD pathway capacity to deplete the ER-stress produced by the accumulation of misfolded proteins is exceeded, the UPR will be triggered, which through different synergistic mechanisms will alleviate the ER-stress (figure 2 and Chapter 2 of this thesis) [18]. These mechanisms are characterized by an increase in the cellular oxidative folding machinery, an enhancement of the proteasome activity together with autophagy activation and a lower protein synthesis rate. If the UPR activation is not enough to compensate ER-stress, the cell will become apoptotic and die [19]. It is the current view that this is the actual way of action of PI for inducing cell death and also the reason why cancerous plasma cells are especially sensitive against this treatment since they are overproducing immunoglobulin for secretion and it is already provoking a basal ER-stress [20]. Recent studies suggest that a modulation of the ER and its associated unfolded protein response (UPR) could be the reason for the adaptation against PIs [20-22].

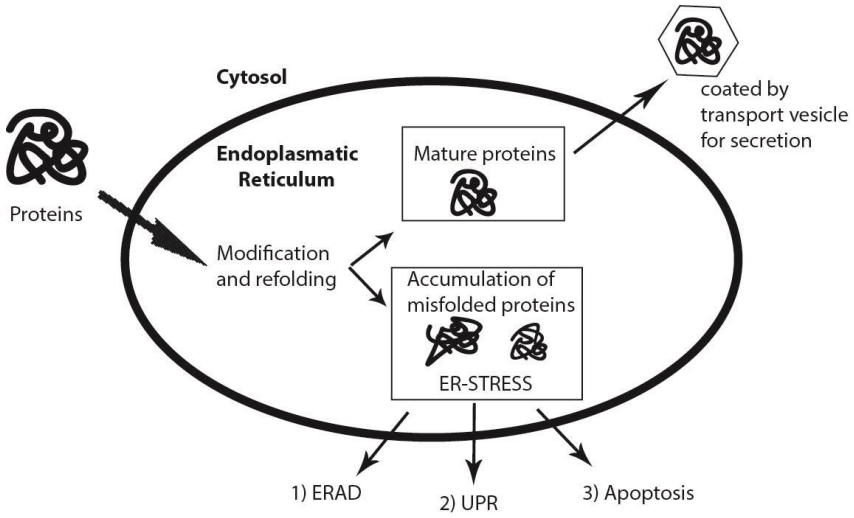

Figure 2. *Schematic representation of ER-stress. Proteins need to be properly folded and in some cases modified prior secretion. This process takes place mainly in the ER. ERAD (1) tags misfolded proteins with poly-ubiquitin chains for proteasomal degradation. If these misfolded or damaged proteins start to accumulate in the ER lumen, the unfolded protein response (UPR) will be triggered (2). The UPR will increase the ERAD capacity and the folding machinery of the ER and at the same time will slow down general protein synthesis in the cell. If UPR activation is not enough for alleviating the stress caused by accumulation of misfolded proteins, cells will enter apoptosis and die (3).*

Activity-based protein profiling (ABPP)

Enzymes are dynamic proteins or protein complexes acting as catalysts in biological reactions. They can be very selective, having a small subset of substrates, or much broader, where they can react with a large variety of substrates. Enzyme activity needs to be tightly regulated, so that only the necessary reactions depending on the cellular needs take place. This can be done with activators, inhibitors, regulatory particles, in some cases by posttranslational modifications (PTMs) or even by keeping the enzyme in a specific cellular compartment where it can only access substrates, which are in the same compartment.

Measuring enzyme activity has always been of great interest for researchers both for fundamental reasons, to expand the knowledge of enzymatic reactions or signaling pathways, and for applied biomedical reasons. Some diseases are directly related to an altered enzyme activity and therefore modulating the activity of some enzymes can be used as treatment against diseases, as is demonstrated in the case of MM by the inhibition of the proteasome. Proteasome activity has been measured mostly by means of

quenched fluorogenic substrates, which become fluorescent after being processed by the proteasome. These substrates allow distinguishing between the three different active sites of the proteasome, β1, β2 and β5, but do not permit the differentiation between the constitutive proteasome and immunoproteasome subunits. In the last 15 years a new method for measuring enzyme activities has been developed, termed activity based protein profiling (ABPP) [23-25]. This technique makes use of tagged inhibitors, called activity-based probes (ABPs), which create a covalent and irreversible bond with the catalytic active site of the enzyme allowing its direct measurement. By means of fluorescent-tagged ABPs the separation and quantification of the 6 different proteasome activities was achieved [26, 27]. ABPP was also used to demonstrate the activity of a new proteasome active subunit, the β5t, which is exclusively expressed in the thymus [28].

ABPs can be used to identify and quantify enzyme activities on gel when bearing a fluorescent tag or for enzyme affinity purification if tagged with an affinity handle such as a biotin moiety. ABPs consist of three parts, an electrophilic trap or warhead, a linker or enzyme targeting moiety and a tag. The warhead is the chemical entity, mostly a nucleophile, which reacts with the active site of the target enzyme creating a covalent bond between the ABP and the enzyme. The linker or backbone is used for enzyme targeting thus making the ABP specific against a single or a broader range or enzymes. This backbone in most cases mimics the enzyme substrate structure or the one of natural compounds found to bind the target enzyme. In some cases the presence of a tag can interfere with the selectivity or potency of the probe and when using it on living cells also to its cellular localization. To avoid these possible caveats the tag can be replaced by a bio-orthogonal handle, generating two-step ABPs [29, 30]. Bio-orthogonal tags are small chemical moieties which are chemically inert under physiological conditions and are able to perform a reaction with another chemical entity under these conditions without interfering with the surrounding [31]. Azide or alkyne groups are the most popular bioorthogonal tags due to their small size, comprising just few atoms, and their highly selective reactions. All these different features are what make ABPP a broadly used technique in the study of a large variety of enzymes. It has been shown to be a robust and reliable concept, which allows quantification of the enzymatic activity or the enrichment of the target enzyme.

Aim and outline of this thesis

The work described in the first part of this thesis (Chapters 3 and 4) is focused on expanding the knowledge about proteasome activity-based probes by in depth

characterization. The use of ABPP and mass spectrometry (MS) in the elucidation of the resistance mechanisms which confer resistance towards proteasome inhibitors in multiple myeloma samples is presented in Chapter 5.

Chapter 2 comprises a literature overview, which covers in more detail the link between the proteasome, proteasome inhibitors and multiple myeloma. The possible adaptation mechanisms will also be briefly discussed with a focus on the UPR and the redox machinery of the cell.

First part of **Chapter 3** presents an overview of two-step proteasome ABPP strategies reported in literature performing different bio-orthogonal reactions. The second part of this chapter describes the characterization of a broad-spectrum ABP and the determination of the unlabeled proteasome fraction after probe exposure.

Chapter 4 describes a screen of 7 different ABPs in mouse and zebrafish tissue extracts.

Chapter 5 provides a study on the mechanisms of adaptation towards proteasome inhibitors in multiple myeloma samples by a combination of ABPP and MS-based proteomics.

Chapter 6 is a summary of the whole thesis and the future prospects for the different chapters.

References

1. Baumeister, W., *et al.*, *The proteasome: paradigm of a self-compartmentalizing protease.* Cell, 1998. **92**(3): p. 367-380.
2. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu. Rev. Biochem., 1998. **67**: p. 425-479.
3. Finley, D., *Recognition and processing of ubiquitin-protein conjugates by the proteasome.* Annu. Rev. Biochem., 2009. **78**: p. 477-513.
4. Tanaka, K., T. Mizushima, and Y. Saeki, *The proteasome: molecular machinery and pathophysiological roles.* Biol. Chem., 2012. **393**(4): p. 217-234.
5. Besche, H.C., A. Peth, and A.L. Goldberg, *Getting to first base in proteasome assembly.* Cell, 2009. **138**(1): p. 25-28.
6. Kloetzel, P.M., *The proteasome and MHC class I antigen processing.* Biochim. Biophys. Acta, 2004. **1695**(1-3): p. 225-233.
7. Groettrup, M., C.J. Kirk, and M. Basler, *Proteasomes in immune cells: more than peptide producers?* Nat. Rev. Immunol., 2010. **10**(1): p. 73-78.
8. Meng, L., *et al.*, *Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity.* Proc. Natl. Acad. Sci. U. S .A., 1999. **96**(18): p. 10403-10408.
9. Adams, J., *et al.*, *Proteasome inhibitors: a novel class of potent and effective antitumor agents.* Cancer Res., 1999. **59**(11): p. 2615-2622.
10. Goldberg, A.L., *Development of proteasome inhibitors as research tools and cancer drugs.* J. Cell. Biol., 2012. **199**(4): p. 583-588.

11. Kisselev, A.F., W.A. van der Linden, and H.S. Overkleeft, *Proteasome inhibitors: an expanding army attacking a unique target.* Chem. Biol., 2012. **19**(1): p. 99-115.
12. Kale, A.J. and B.S. Moore, *Molecular mechanisms of acquired proteasome inhibitor resistance.* J. Med. Chem., 2012. **55**(23): p. 10317-10327.
13. McBride, A. and P.Y. Ryan, *Proteasome inhibitors in the treatment of multiple myeloma.* Exp. Rev. Anticancer Ther., 2013. **13**(3): p. 339-358.
14. Ri, M., *et al.*, *Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress.* Leukemia, 2010. **24**(8): p. 1506-1512.
15. Lichter, D.I., *et al.*, *Sequence analysis of beta-subunit genes of the 20S proteasome in patients with relapsed multiple myeloma treated with bortezomib or dexamethasone.* Blood, 2012. **120**(23): p. 4513-4516.
16. Politou, M., *et al.*, *No evidence of mutations of the PSMB5 (beta-5 subunit of proteasome) in a case of myeloma with clinical resistance to Bortezomib.* Leuk. Res., 2006. **30**(2): p. 240-241.
17. Ron, D. and P. Walter, *Signal integration in the endoplasmic reticulum unfolded protein response.* Nat Rev. Mol. Cell Biol., 2007. **8**(7): p. 519-529.
18. Walter, P. and D. Ron, *The unfolded protein response: from stress pathway to homeostatic regulation.* Science, 2011. **334**(6059): p. 1081-1086.
19. Tabas, I. and D. Ron, *Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress.* Nat. Cell Biol., 2011. **13**(3): p. 184-190.
20. Gu, J.L., *et al.*, *Differentiation induction enhances bortezomib efficacy and overcomes drug resistance in multiple myeloma.* Biochem. Biophys. Res. Commun., 2012. **420**(3): p. 644-650.
21. Oyadomari, S., *et al.*, *Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload.* Cell, 2006. **126**(4): p. 727-739.
22. Niewerth, D., *et al.*, *Molecular basis of resistance to proteasome inhibitors in hematological malignancies.* Drug Resist. Updat., 2015. **18**: p. 18-35.
23. Willems, L.I., H.S. Overkleeft, and S.I. van Kasteren, *Current developments in activity-based protein profiling.* Bioconjug. Chem., 2014. **25**(7): p. 1181-1191.
24. Cravatt, B.F., A.T. Wright, and J.W. Kozarich, *Activity-based protein profiling: from enzyme chemistry to proteomic chemistry.* Annu. Rev. Biochem., 2008. **77**: p. 383-414.
25. Evans, M.J. and B.F. Cravatt, *Mechanism-based profiling of enzyme families.* Chem. Rev., 2006. **106**(8): p. 3279-3301.
26. Gu, C., *et al.*, *Proteasome activity profiling: a simple, robust and versatile method revealing subunit-selective inhibitors and cytoplasmic, defense-induced proteasome activities.* Plant J., 2010. **62**(1): p. 160-170.
27. Verdoes, M., *et al.*, *A panel of subunit-selective activity-based proteasome probes.* Org. Biomol. Chem., 2010. **8**(12): p. 2719-2727.
28. Florea, B.I., *et al.*, *Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t.* Chem. Biol., 2010. **17**(8): p. 795-801.
29. van der Linden, W.A., *et al.*, *Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study.* Bioorg. Med. Chem., 2012. **20**(2): p. 662-666.
30. Willems, L.I., *et al.*, *Bioorthogonal chemistry: applications in activity-based protein profiling.* Acc. Chem. Res., 2011. **44**(9): p. 718-729.
31. Shieh, P. and C.R. Bertozzi, *Design strategies for bioorthogonal smart probes.* Org. Biomol. Chem., 2014. **12**(46): p. 9307-9320.