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The use of activity based protein profiling to study proteasome biology

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Chapter 6: Summary and Future Prospects

The work described in this thesis focuses on the characterization of proteasome directed activity-based probes (ABPs) as well as on the adaptation mechanisms that make multiple myeloma derived cell lines resistant against proteasome inhibitors (PIs). **Chapter 1** comprises a general introduction to the ubiquitin proteasome system (UPS) and to the techniques and tools used in this thesis to its study. The UPS is the main pathway for cellular protein degradation in eukaryotic cells. In the UPS pathway ubiquitin marks proteins destined for destruction by the proteasome, the actual protein degradation machinery. Partial inhibition of the proteasome has been approved as a treatment for two types of blood cancers (multiple myeloma and mantle cell lymphoma) but in most cases patients relapse and become insensitive against proteasome inhibition regimes.

Chapter 2 presents a literature review and entails an in-depth analysis on the UPS, its inhibition and its relation with cellular redox homeostasis. PIs are used in the clinic for treatment of hematopoietic malignancies. They induce endoplasmic reticulum (ER) and oxidative stress, disruption of signaling pathways, mitochondrial dysfunction and eventually cell death caused by apoptosis. PIs designated as clinical candidates include natural product derivatives and compounds developed by rational design and feature a wide diversity of structural elements. Research in recent years has brought a deepened insight into the molecular mechanisms of PI induced apoptosis. However there are some paradoxes and controversies in the literature. In chapter 2 the advances and uncertainties, in particular on the time course events that makes cells commit to apoptosis are discussed. Also some mechanisms of evolved PI resistance are presented, and speculations on the difference in sensitivity between cell or tumor types are brought forward.

Increased understanding of the systems biology at mRNA and protein level and the kinetics behind the interaction between proteasome inhibitors and cells is imperative. Design and synthesis of subunit specific inhibitors for each of the seven known proteasome activities and for the enzymes associated to proteasomes will aid in unraveling biology of the UPS in relation to ER stress, ROS production and apoptosis and will generate leads for therapeutic intervention.

The first part of **Chapter 3** describes different bio-orthogonal strategies to label the proteasome subunits by means of two-step activity-based protein profiling (ABPP). Because the reporter groups can alter the properties of ABPs, introducing bio-orthogonal

handles instead of readout tags allows performing first the proper binding of the ABP to the enzyme and a posterior addition of the reporter group in a second reaction. Bio-orthogonal handles are small chemical moieties which are inert in complex biological environments. The bio-orthogonal reactions can be performed both *in situ* and *in vitro*. In fact, some of the bio-orthogonal ligations can be performed simultaneously. Alkynes and cyclooctynes respective ligations show background labeling, which in the case of cyclooctynes is very large, suggesting that cyclooctyne ligations are not truly bio-orthogonal. These extra bands should be analyzed in more detail, for example by in-gel digestion coupled to LC-MS analysis or by a pull-down experiment. Finding the biological groups that react with these molecules could be beneficial for developing new bio-orthogonal handles or for inhibitors and probes development. Although the pool of tools in bio-orthogonal chemistry has increased considerably in the last decade, there is still room for improvement as many chemical reactions might also be suitable thus expanding the tools for researchers. Ideally, finding new reactions which are orthogonal between them and also with biological samples would be the best. This would increase the tools for a simultaneous labeling of different proteins or other constructs.

The second part of **Chapter 3** is a technical study about the capacity of an ABP to label the whole pool of active proteasomes from human and murine cell lines both *in vitro* and *in situ*. The optimal conditions for the labeling are determined, incubation with 0.5 µM for 1 h *in vitro* and 4 µM for 4 h *in situ*. Interestingly these conditions were the same for both cell lines studied, namely AMO-1 and B3/25. The unlabeled fraction after labeling with the probe was found to be very small (1-10%) in all the cases except for mice cell lysates where the percentage increased up to 30%. This large difference between the unlabeled proteome *in vitro* and *in situ* suggests that it might be caused by a systematic error while performing the experiments. The experiments in murine cells need to be repeated in order to validate the here obtained results. Performing this screen with different ABPs and on different species may give an indication on the specificity and potency of not only the probes but also the inhibitors, and could help in the development of new and more potent and, perhaps also, organism selective probes and inhibitors.

Chapter 4 describes a screening of 7 different proteasome-directed ABPs in mouse and zebrafish tissue extracts. The ABPs used in this study vary from subunits specific probes to broad-spectrum ones. Their application in human samples has been validated previously and here it is shown that these are also suitable for their use in mouse and zebrafish, two species broadly used in research due to their similarity to human biology. Although all ABPs retain their subunit specificity with a low lose in potency (all working in the low micromolar range, 0.1-3 µM) in both organisms, some observations can be made. Probe **6**, a β5-selective probe, does not give a proper separation of the mouse β5 and β5i subunits

when running on SDS-PAGE as it does with the human counterparts. Only when performing a 2D gel electrophoresis the separation and visualization of the two subunits is achieved. Possibly replacement of the reported tag by a different fluorophore might allow its separation on a regular SDS-PAGE. In the case of zebrafish extracts, the use of BODIPY(FL)-bond ABPs is not recommended when labeling the β2 or β2i subunits due to an endogenous green fluorescent protein which runs slightly higher than these subunits in the gel. The presence of extra bands next to the β2 subunit when labeling zebrafish brains or full fish extracts with probes **2** and **7** was not expected, since these bands are not visible in human or mouse samples. Further research needs to be done on this, since it could be that the probes are allowing the separation of different post-translationally modified β2 subunits or they could also be off-targets. Probably an in-gel digestion procedure coupled to a mass spectrometry analysis will be an appropriate solution to identify those unexpected bands.

The large changes in probe potency in the different organisms tested indicates that there might be a window where the probes or inhibitors might be selective towards one organism but not another. The development of organism selective proteasome ABPs and inhibitors might be beneficial not only for fundamental but also for clinic research. This would allow for example the specific proteasome inhibition of the pathogen but not of the host in infections. The development of these types of inhibitors might be also useful in the food and agriculture industry, where plagues could be avoided by compounds that only target the organism causing the plague but not the plant itself and neither individuals who might consume these afterwards.

Chapter 5 reports on the characterization of two PI resistant cell lines by means of ABPP and quantitative proteomic techniques. This study shows that adaptation towards proteasome inhibition is not only independent from the type of inhibitor but also from point mutations in the β5 active site binding pocket, which have been previously reported in cell lines studies but not in patients with relapse or refractory myeloma. The significant changes in the proteome of two resistant subclones of a multiple myeloma cell line were characterized and compared to their progenitor cell line. When combining the identified proteins into clusters according to their biological function, both cell lines showed the same biological pathways being altered compared to the wild type cells. This, together with to the fact that around 50% of the proteins found in the analysis were shared between both adapted subclones, is indicating that a complex biological network rearrangement is driving the adaptation towards proteasome inhibition induced cytotoxicity. The data point towards some potential druggable targets including antioxidant enzymes and ion pumps, which blockade might induce apoptosis in the adapted cell lines. In figure 1 a model of the obtained results that characterize the

resistant adaptation mechanisms is presented. All these different biological process could be a suitable target for alternative treatment against PI resistant cells.

In order to validate these targets and in general the global adaptation mechanisms found in our data, this type of analysis should be repeated with different disease-stage cell lines and if possible it would be ideal if this is done with patient material. In fact, the global procedure of the analysis can be extrapolated and used in the study of any drug resistance development in other diseases or even just in the differentiation characterization between two cell types. This technique if applied in the clinic could drive towards patient-based therapies, by finding the specific and optimal altered signaling pathway to target.

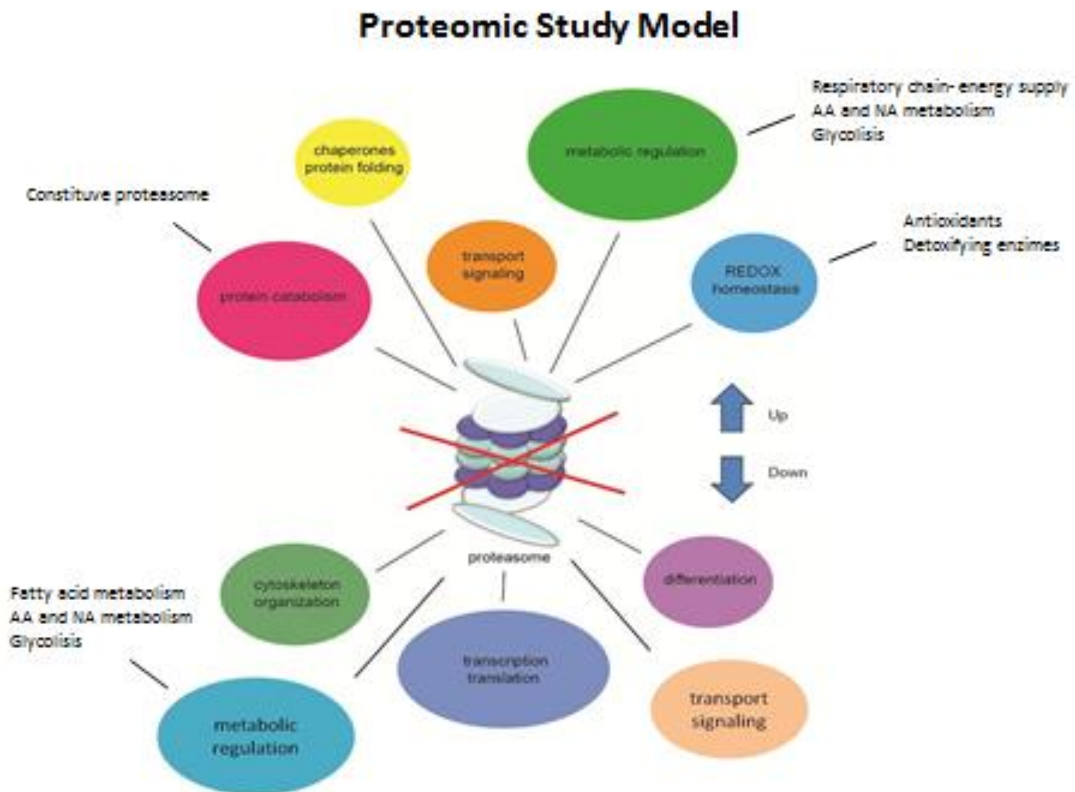

***Figure 1**. Model showing the different biological processes inducing proteasome inhibitor resistance in our cell lines adaptation model. The size of the different biological bubbles is related to the proteins found in each cluster.*

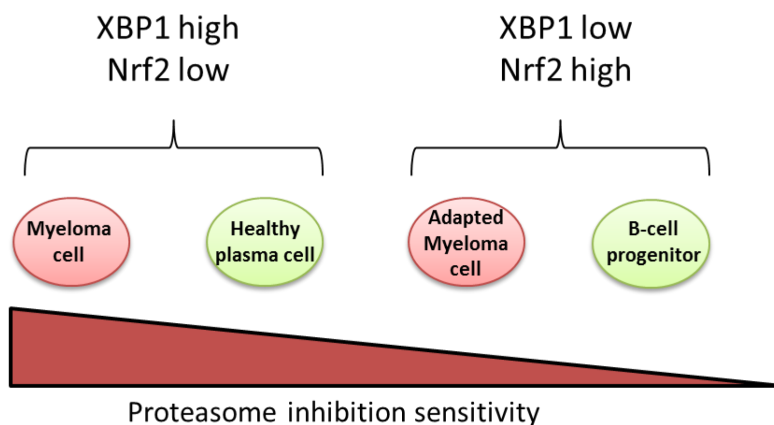

Figure 2. *Proposed model for proteasome inhibition adaptation.*

Fully functional plasma cells are characterized by a high XBP1 and a low Nrf2 expression, while their progenitors are characterized by the opposite, a low XBP1 and a high Nrf2 expression. The high need of plasma cells for protein quality control makes them potentially sensitive for proteasome inhibition (figure 2). The malignant plasma cells have an increased protein synthesis rate, making them more sensitive against PI induced apoptosis. It can be hypothesized that the adaptation results in a loss of XBP1 (actually a factor revealed by the presented data) and an increase of the transcription factor Nrf2. These changes trigger cellular de-differentiation and thus making the cells insensitive against proteasome inhibition. The transcription factor Nrf2 is essential for antioxidant response in all cell types, by inducing the expression of antioxidants like NQO1, one of the top proteins found overexpressed in the adapted clones. It is also known for the induction of constitutive proteasome subunits, another feature found in our analysis. This data is suggesting that Nrf2 transcriptional activation or overexpression might trigger the response mechanisms, at least in some extend, but this still needs to be validated. To check whether Nrf2 is essential for PI adaptation, it could be attempted to develop PI resistance in a knocked down cell line. Alternatively, the use of Nrf2 inhibitors or activators should also indicate if this transcription factor is needed for the adaptation process.