Summary of Małgorzata Giżyńska's doctoral dissertation:

"The use of the *C*-terminal sequence of the Blm10 protein to obtain effective stimulators of human 20S proteasome activity"

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Incorrect functioning of the proteasome-ubiquitin system leads to a disturbance of protein homeostasis, causing malfunctioning of the cell cycle or expression of genes mechanisms, and the accumulation of damaged proteins in cells, which can ultimately lead to disease states. Proteasome seems to be an attractive target in the design of potential therapeutic agents for cancer, cardiovascular, neurodegenerative and autoimmune diseases. Currently, the most exploited concept is the use of the enzyme's inhibitors in cancer therapy. In recent years, three proteasome inhibitors have been approved for the treatment of multiple myeloma and lymphoma, and many others are undergoing clinical trials. Unlike inhibitors, compounds that can increase proteasome activity are few and poorly understood. Such compounds could find application in the therapy of diseases, in which the observed reduced activity of the proteasome contributes to the accumulation of toxic, oligomeric forms of proteins in neurons, thus disrupting their functioning, and eventually leading to their death.

Currently, proteasome activators are sought mainly by screening compound libraries. So far, several small molecule compounds have been discovered that directly or indirectly stimulate proteasome activity. It was observed that some of them were able to inhibit aging processes, slow down the development of neurodegenerative diseases (e.g. in cellular models of Alzheimer's disease), as well as increase cell resistance to oxidative stress. However, they were usually broad-spectrum compounds that would have to go a lengthy process of structure optimization in order to act more selectively and not cause serious side effects. Another possible approach to the design of new modulators is the use of natural proteasome activators: 19S, 11S

or PA200 / Blm10, as a base structures. However, despite the resolved structures of 20S complexes with protein activators, the activation mechanism itself has not been fully understood, which hinders the design of effective modulators.

The main goal of my research was to obtain effective stimulators of human proteasome activity, designed based on Blm10 and Blm-pep activators. Our team has been conducting research on allosteric modulators of proteasome activity for several years, which resulted in the 14-residue Blm-pep activator that stimulated all h20S peptidases several times. It was designed based on the fragment through which Blm10 binds to the proteasome, and comprised six *C*-terminal residues of this fragment. In the solved crystal structure of the complex between yeast 20S and Blm-pep only last five residues were visible in the electron density, which suggests that only this fragment permanently binds to the enzyme.

In the first stage of my work, I examined the importance of the *N*-terminal region of the Blm-pep. For this purpose, I synthesized eleven shorten analogs of the modulator and analyzed their stimulating capacity. The obtained results allowed to state that despite the lack of the *N*-terminal part of Blm-pep in the electron density, it is indispensable for the effective activation of h20S, but the nature of its interaction with the enzyme is probably only transient.

In order to obtain more effective stimulators of h20S proteasome activity I performed optimization of the Blm-pep structure based on molecular modeling using the data from the crystal structures of y20S complexes with Blm10 and Blm-pep and the crystal structure of h20S proteasome, The results obtained indicate that an effective proteasome activator should have an acidic and aromatic residue in positions 8 and 9, as well as at least one aromatic residue at the *N*-terminus. In contrast, the presence of an acidic residue in position 1 or in the linker between the *N*- and *C*-terminal segments significantly reduces the stimulating capacity of Blm analogs towards ChT-L and PGPH peptidases. Studies have also shown that the HbYX motif cannot be modified, even if this modification potentially could allow more efficient binding in the α pocket. Substituting tyrosine residue from this motif with aspartic acid led to a complete loss of the activating capacity of the compound. By introducing modifications to the *N*-terminal region of the peptide, modulators acting selectively on the T-L peptidase have been obtained.

All activators were susceptible to degradation by h20S. To check whether degradation of the activator enhances the stimulating effect by the cross-talk of the active and allosteric sites, I have attempted to obtain proteolytically stable modulators. Unfortunately, the introduction of amino acids with unnatural side chains, as well as modifications altering the secondary structure of the compounds compromised the modulators activity. Peptide bond modifications were better tolerated. I managed to obtain two compounds with increased resistance to degradation

by h20S, which did not lose the ability to activate the enzyme and can be the starting sequences for further modifications.

The effectiveness of selected Blm analogs has been tested using model protein substrates. These compounds were able to stimulate protein degradation only in the presence of an agent (SDS) relaxing the conformation of the 20S catalytic core. This agrees with the literature data, which reports that wide and permanent opening of the gate leading to the catalytic sites is possible only as a result of the binding motifs anchoring in at least four α pockets. The signal coming only from one pocket, without additional support, does not allow the gate to be opened wide enough to allow protein particles to enter the catalytic channel.

For selected compounds the activity tests using cell models of amyotrophic lateral sclerosis (ALS) and of Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases were performed. Compound **20** stimulated proteasome activity in AD, PD, HD and ALS cell models, while **18** only in the ALS model. These modulators also stimulated the degradation of proteins with disordered structure (SOD, tau), without affecting the level of a folded control protein (β-actin), which suggests that tested compounds interact only with the 20S proteasome, not the 26S, so they should not cause uncontrolled enhancement of total proteolysis conducted by the UPS system.

A separate goal of my work was to identify the binding site/s of allosteric activators in the structure of the human 20S proteasome. This task was executed through chemical cross-linking in combination with mass spectrometry. I introduced cross-linking residues, such as 2,4-dihydroxyphenylalanine or benzoylphenylalanine (Bpa), to the sequence of selected Blm-pep analogs. After formation of the covalent adduct of h20S with the modulator, the ligand-linked proteasome subunits were identified by mass spectrometry. The intact-protein MS revealed Blm analogs bound to both α and β subunits. For all Blm analogs containing a Bpa cross-linking residue, the constantly recurring site of interaction was α3 subunit. To identify the exact sites of interaction, the complex, formed through photo-crosslinking of a modulator to h20S, was subjected to enzymatic digestion. The tandem MS analysis of this digest demonstrated that one of the tested modulators bound in the α5-α6 pocket, and the other was found in the pockets α4-α5 and α5-α6.