SUMMARY

Healthy cells are characterized by the presence of a complex, well-functioning network consisting of proteolytic enzymes and chaperones that maintain a state of homeostasis. The proteasomal system is responsible for degradation of redundant proteins marked by a ubiquitin tag, intrinsically disordered proteins or proteins that have been damaged under conditions such as oxidative stress. During aging, both external and endogenous factors influencing protein damage increase. Such an age-dependent decline in the ability of cells to maintain proteostasis is believed to be a major contributor to age-related cellular dysfunctions, including the deposition of toxic protein aggregates, which have been closely linked to a number of diseases, particularly those associated with nervous system malfunctions. It has also been reported that the disassembly of the 26S proteasome progresses with age, with a concomitant increase in the amount of the 20S proteasome; however, increased 20S activity was not observed. Therefore, it can be concluded that aging drives the formation of a new pool of the 20S proteasome, which is not bound to its specific regulators such as 19S, 11S or Blm10 protein. For this reason, treatment involving the introduction of exogenous modulators of its activity appears to be a promising way to prevent the accumulation of toxic proteins. To date, researchers' attention has focused on the search for 20S activators mainly among small-molecule compounds, which appear to be more attractive pharmaceuticals than peptides due to their greater stability and bioavailability. On the other hand, one should keep in mind their low selectivity and often high toxicity. Therefore, in my studies I focused on searching for peptide stimulators based on native proteins that regulate 20S proteasome activity and verified their potential to stimulate the enzyme *in vitro* and *in cellulo*.

In the first stage of my work, I focused on sequences derived from the *C*-terminus of the Rpt subunits of the 19S regulator, which are reported to be actively involved in opening the proteasome gate. Based on activity results, of the 6 compounds synthesized, the Rpt5(8) peptide turned out to be the most promising, so in further studies I focused on optimizing its sequence. In the course of my research, I verified the impact of individual amino acid residues in this peptide by elongating its sequence, using the alanine scanning method and synthesizing a library of compounds with the side chains of different character. These studies led to the conclusion that the optimal length of the activator is 10 residues (compound Rpt5(10)), with basic residues most preferred at positions 1, 2 and 4 of this peptide, while position 7 seems to be the most liberal in terms of accepted residues. In the next step, I tested the stability of the

Rpt5(10) peptide in the presence of the human 20S proteasome and in human plasma. After 1h of incubation with h20S, this analog remained undigested at about 40%, while after just 10 min of incubation with plasma, a little over 30% remained. For this reason, I synthesized 6 new analogs based on the structure of the Rpt5(10) peptide, which resulted in the most potent activator - the Rpt5(Nle8) compound. Unfortunately, this peptide did not show stability higher than the parent peptide. In contrast, I observed stronger resistance to degradation in peptides in which position 4 was modified (compounds Rpt5(Abu4), Rpt5(βAla4) and Rpt5(AzrN)).

In view of obtaining promising h20S activators, I decided to check if they would be able to show a similar effect in living cells. Therefore, I tested the ability of the Rpt5(10) peptide to penetrate the cell membrane. This peptide, having 3 basic lysine residues at its *N*-terminus, could act similarly to cationic CPPs, however, it penetrated the membrane only to a small extent. Thus I checked whether attaching the Tat(48-57) sequence would make the peptide capable of penetrating cells. As it turned out, such a modified sequence successfully overcame the cell membrane barrier and, in addition, more strongly stimulated the ChT-L peptidase of the isolated 20S proteasome. Encouraged by the results of these studies, I synthesized 3 more analogs, containing a penetrating sequence attached to two of the most potent activators obtained: Rpt5(K4) and Rpt5(Nle8), as well as the peptide Rpt5(D6), which exhibited no stimulatory potential. I tested all four compounds for stimulation of ChT-L proteasome activity in HEK 293T cell lysate. The outcome of these assays correlated with previous results obtained on the isolated enzyme: the compounds Tat-Rpt5(10), Tat-K4 and Tat-Nle8 activated the enzyme even more effectively than their analogs without the attached CPP sequence. Again, the strongest modulator was the peptide containing Nle8 residue. In addition, it was possible to rule out that the Tat(48-57) sequence was responsible for the activating effect, as both the analogous compounds: lacking the activator sequence Tat-LLVY and Tat-D6 peptide were unable to stimulate the enzyme to degrade the substrate.

The final step in this part of the study was to check if the obtained compounds would be able to activate the proteasome in live HEK 293T cells. For this purpose, I used the TAS3 probe, which has good resistance to other cellular enzymes. The results obtained confirmed that the Tat-Nle8 peptide is the most potent of the proteasome modulators. Moreover, it was stable enough to activate the proteasome despite a number of proteolytic enzymes present in living cells. This can be considered a success as peptides are often disqualified as drugs due to their low proteolytic stability and lack of ability to penetrate the cell membrane. In addition, the

compounds did not show a large cytotoxic effect against the HEK 293T line, which also makes them promising candidates for further research.

The second part of my work concerned peptides derived from the Blm10 protein. These compounds were designed based on molecular modeling to determine the most optimal linker length connecting the *C*- and *N*-terminal segments, as well as the *N*-terminal sequence allowing interaction with the α5 subunit of the 20S proteasome. Studies using peptide substrates determined that the linker length should be 4 amino acid residues. In order to facilitate the synthesis and increase the solubility of the peptides, I exchanged them into one Peg2 moiety. The *N*-terminal region of the designed modulator consisted of the sequence: Lys-Asn-Ser-Asn (peptide M2-6).

Six more compounds were then designed by docking the *C*-terminus of the Blm10 protein into the pockets formed by the α subunits of the proteasome, and determining the sequences of the four *N*-terminal amino acids that could potentially interact with the environment in each pocket (compounds M2-1, M2-2, M2-3, M2-4, M2-5, M2-7). Studies performed using a longer peptide substrate (LFP), which, due to its weaker digestion by the latent proteasome, better differentiates potential activators, showed that compound M2-6 designed to interact with the α5 subunit remains the strongest modulator. For this reason, I decided to optimize the sequence of this particular peptidomimetic. For this purpose, I substituted Lys1 with an acidic residue (Glu) and a residue having a neutral hydrophobic side chain (Leu). These peptides more strongly than M2-6 stimulated only the trypsin-like activity of the proteasome, for the other substrates the effect was comparable or weaker than the parent compound. The strongest modulators were the peptides M2-10 and M2-20. The former had an unnatural homoarginine residue in first position and aspartic acid in position 6, where leucine was natively present. This compound most strongly stimulated the chymotrypsin-like activity of the proteasome. M2-20 analogue differing from the starting M2-6 peptide by only one residue, Lys4 instead of Asn4, most strongly stimulated the proteasome to digest the longer LFP substrate and the substrate of T-L peptidase. The compound that completely lost its ability to activate the enzyme turned out to be the M2-9 peptide, which had a D-tyrosine at position 10, which presumably caused blocking of the *C*-terminal binding in the α pocket of the proteasome.

Since short fluorogenic substrates do not fully reflect the role the proteasome plays in cells, I decided to check if the compounds I obtained would be able to activate the enzyme for protein digestion. Since the 20S proteasome lacks an attached 19S regulatory moiety, and is

therefore unable to unfold large, structured proteins, it is mainly responsible for digesting damaged or intrinsically disordered proteins. To assess the enzyme's ability to interact with the proteins selected for activity assays, I performed affinity tests using microscale thermophoresis. These tests allowed to observe the binding of oxidized proteins and native (belonging to the group of intrinsically disordered proteins) α-synuclein to the 20S proteasome. In contrast, a native enolase, possessing a folded structure, showed no affinity for 20S. The modulators I obtained were also unable to stimulate the enzyme to digest this protein, however, they readily stimulated it to digest oxidatively damaged proteins and native synuclein. It gives hope for future use of such peptides as drugs to enhance the removal of only those proteins with aggregation potential. Of particular note is the peptide M2-12, which strongly accelerated the degradation of both native and oxidized α-synuclein and oxidized enolase. Also promising is the compound M2-2, which strongly stimulated the proteasome to digest native α-synuclein.

I also subjected the aforementioned peptides M2-2, M2-12 and compound M2-10 to cytotoxicity tests. The cell-damaging effect was visible only at high concentrations of the modulators (50 μM). Despite the relatively poor degradation resistance in human plasma (about 20-30% undigested peptide after 15 min of incubation), all 3 compounds were able to activate the proteasome in cell lysate.