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SUMMARY OF DOCTORAL DISSERTATION

„*Characteristics of human proteasome specificity by combinatorial chemistry methods* ”

Proteasome is a multi-catalytic protein complex (EC 3.4.25.1) found in all living organisms, in both *Eucaryota* and *Procaryota*. It is responsible for degradation of damaged, modified and misfolded proteins or proteins that have become expendable. As proteasome is involved in many fundamental biological processes, like the cell cycle regulation, apoptosis or angiogenesis, even the smallest disruption in its activity may cause very serious physiological consequences, including cancer diseases. The mammalian proteasome 20S catalytic core contains two sets of three catalytically active β subunits, which display a different substrate specificity, namely β1 (caspase-like) cleaves after acidic residues, β2 (trypsin-like) cleaves after basic residues and β5 (chymotrypsin-like) cleaves after bulky, hydrophobic residues.

The first aim of my dissertation was the synthesis of novel, highly selective and sensitive human proteasome 20S substrates, separately for each enzymatically active β subunits. It is worth to mention that I analyzed substrates in the region so far undiscovered. In world literature I have not found any information about proteasome's specificity in residues carboxy-terminal to the scissile peptide bond (so called prime side according to nomenclature of Schechter and Berger).

Firstly, I designed and synthetized three tetrapeptide libraries, which consisted of fluorogenic and chromogenic human proteasome's 20S substrates. The general formula of each peptide library was as follows: ABZ-X_4-X_3-X_2-X_1-ANB-NH_2, wherein positions of X_4, X_3 and X_2, the set of all amino acid proteinogenic except Cys were placed. The X_1 position was optimized separately for each specificity: for chymotripsyn-like Tyr and Phe residues were introduced, for trypsin-like it was Arg and Lys and for caspase-like specificity Asp and Glu residues were placed. ABZ (2-amino-benzoic acid), and ANB (2-amino-3-nitro-benzoic acid) at *N*- and *C*-termini, respectively, serve as a donor / acceptor pair, which shows the distance fluorescence resonance energy transfer (FRET). The synthesis (spilt and mix method) was carried out manually used the Fmoc chemistry on TentaGel S RAM resin as a solid support. The iterative deconvolution in solution of each library allowed me to obtained 3 substrates optimized in non-prime positions:

- chymotrypsin-like subunit: ABZ-Val-Val-Ser-Tyr-ANB-NH_2;
- trypsine-like subunit: ABZ-Val-Val-Ser-Arg-ANB-NH_2;
- caspase-like subunit: ABZ-Ile-Leu-Met-Asp-ANB-NH_2.

In the next step of my research the peptide resulting from above described studies was incorporated into internal quenched library with general formula: ABZ-peptide-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2, where in all positions the set of all proteinogenic amino acids except Cys were used. The ABZ (2-amino benzoic acid) and Tyr(3-NO_2)-NH_2 (amide of 3-nitro-L-tyrosine) at the *N*-and *C*-termini of the synthesized peptides served as donor/acceptor FRET pair and peptides is a sequence that was obtained during earlier studies. For chymotrypsin is Val-Val-Ser-Tyr sequence, for trypsin Val-Val-Ser-Arg peptide and caspase the sequence is Ile-Leu-Met-Asp. Deconvolution of such libraries resulted in peptide sequences:

- chymotrypsin-like subunit: ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO_2)-NH_2;
- trypsin-like subunit: ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2,
  ABZ-Val-Val-Ser-Arg-Ala-Phe-Phe-Tyr(3-NO_2)-NH_2;
- caspase-like subunit: ABZ-Ile-Leu-Met-Asp- Ala-Met-Gly-Tyr(3-NO_2)-NH_2.

Moreover, the primary sequences ABZ-Val-Val-Ser-X_1-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2 and ABZ-Val-Val-Ser-X_1-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 were used to test different structural derivatives of Arg and Tyr residue in X_1 position. I synthesized 24 analogs among which the one with 4-guanidino-L-phenylalanine turned out to be the best in interaction with proteasome. All peptides and peptide libraries were characterized by means of mass spectrometry (MS) and high performance liquid chromatography (HPLC) analysis.

In the next part of my dissertation, I decided to measure proteasome activity level in biological samples. During experiments I was using substrates with the best kinetic parameters. It is stated that proteasome 20S is commonly detected in cancer samples, so I tested one hundred samples of biological material (urine and serum) originating from patients diagnosed with cancer (69) and healthy volunteers (32). In serum samples no clear proteolytic patterns were observed for any of the tested compounds. Such an observation was rather disappointing, however serum is a rich source of proteolytic enzymes, so it was expected for a relatively long peptide with multiple potential cleavage sites. A different situation was observed in the urine samples. In most (85%) of the cancer samples (60 out of 69) there was a detectable increase in fluorescence. The activity of the proteasome in the samples derived from healthy donors was found to be at or below the detection level.

The final selected sequence, ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(NO_2)-NH_2, is effectively cleaved by this multicatalytic complex and using this peptide I was able to measure the proteasomal activity in human urine samples from individuals diagnosed with bladder cancer. This compound was also submitted for patenting.