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„Synthesis strategy development for ubiquitin derivatives with unnatural amino acids as specific and selective tools for investigation of deubiquitinating enzymes"

Ubiquitin (Ub) is a small eukaryotic regulatory protein comprised of 76 amino acid residues. During the ubiquitination event, Ub is covalently attached to a substrate protein. Ubiquitination is involved in processes such as proteasomal degradation of proteins, gene expression and cell cycle regulation, or DNA repair. Ub-dependent signalling is regulated by deubiquitanting eznymes (DUBs). These proteases hydrolyze bonds between Ub and substrate proteins. There are around 100 DUBs in humans and disregulation of many of them is related to cancer development, neurodegenerative diseases, or infectious diseases. Knowledge of detailed roles of deubiquitinases remains elusive due to the lack of selective chemicals tools to study these enzymes.

Goal of the dissertation was the synthesis of ubiquitin derivatives with unnatural amino acids in the C-terminal region of the peptidic sequence of ubiquitin, that are selectively reactive towards human UCH-L3 and viral MERS-CoV PL^{pro} enzymes. In the first part of the project, a library of tetrapeptide fluorogenic substrates was synthesized. The structure of the substrates in the library was Ac-L-R-X-G-ACC, where 'X' is one of the 128 natural or unnatural amino acid residues. The library and a HyCoSuL P4-P3 library were used to investigate substrate specificities of UCH-L3 and MERS-CoV PLpro at P4-P2 positions. Based on the results, tetrapeptidic fluorogenic substrates with unnatural amino acids were designed and synthesized. Substrate specificity constants k_{cat}/K_M for the substrates were determined. Next, selectivity

of the substrates towards the investigated enzymes was determined. Sequences of the best substrates were incorporated into the C-terminus of Ub derivatives, that were synthesized using solid phase peptide synthesis (SPPS) and ligation-desulfurization strategies. Four substrates were synthesized:

1. Ub-ACC – native Ub with C-terminal ACC group,
2. Ub.M2-ACC – Ub derivative with C-terminal Tle-Phg-Gly-Gly-ACC sequence for MERS-CoV PL^{pro};
3. Ub.S1-ACC – Ub derivative with C-terminal Cha-Arg-Abu-Gly-ACC sequence for UCH-L3;
4. Ub.S2-ACC – Ub derivative with C-terminal D-Arg-Phe(guan)-Ala-Gly-ACC sequence for UCH-L3.

Selectivity and kinetic parameters (k_{cat}, K_M, k_{cat}/K_M) of the substrates towards UCH-L3 and MERS-CoV PL^{pro} were determined. Substrates were selective and specific towards the enzymes.

Next, four activity-based probes (ABPs) with structures based on Ub molecule were synthesized. The ABPs were synthesized using SPPS and ligation-desulfurization techniques:

1. biot-6-ahx-Ub-VME – biotinylated Ub derivative with native sequence and C-terminal VME group;
2. biot-6-ahx-Ub.M2-VME – biotinylated Ub derivative for MERS-CoV PL^{pro} with C-terminal Tle-Phg-Gly-Gly-VME motiff;
3. biot-6-ahx-Ub.S1-VME – biotinylated Ub derivative for UCH-L3 with C-terminal Cha-Arg-Abu-Gly-VME motiff;
4. biot-6-ahx-Ub.S2-VME – biotinylated Ub derivative for UCH-L3 with C-terminal D-Arg-Phe(guan)-Ala-Gly-VME motiff.

Inhibitory properties of the ABPs towards MERS-CoV PL^{pro} and UCH-L3 were determined. Next, ABPs were used for selective detection of the investigated enzymes in cell lysates. The experiment confirmed selective and sensitive labeling of MERS-CoV PL^{pro} and UCH-L3 in lysates from A-431, HeLa, and HEK-293T cell lines.

In conclusion, incorporation of unnatural amino acids within the C-terminus of ubiquitin provides specific and selective recognition of these Ub derivatives by deubiquitinases. Presented strategy enabled synthesis of selective and specific substrates and ABPs for investigation of human UCH-L3 and viral MERS-CoV PL^{pro} enzymes. The hypothesis was confirmed using recombinant enzymes and cell lysates.