PhD Thesis: "Investigation of the role of calpain-1 in the process of pyroptosis using tailored chemical tools" by Natalia Horbach, MSc

SUMMARY

Calpain-1, a member of the calcium-dependent cysteine protease family, requires calcium ions for activation and is involved in various cellular processes. Structurally, calpain-1 is a heterodimer composed of a large 80 kDa catalytic subunit and a smaller 28 kDa regulatory subunit. The catalytic subunit contains domains responsible for calcium binding, proteolysis, and interaction with the regulatory subunit, which in turn contains EF-hand motifs that bind calcium. Calpain-1 exerts its enzymatic activity through the precise cleavage of specific substrates, thus regulating diverse cellular functions. Notable calpain-1 substrates include cytoskeletal proteins (e.g., spectrin, talin), signaling molecules (e.g., protein kinase C, focal adhesion kinase), and membrane proteins. This protease plays a critical role in cytoskeletal remodeling, signal transduction, and cellular motility.

In my PhD project, I focused on the role of calpain-1 in cell death, particularly in apoptotic and pyroptotic processes. During apoptosis, calpain-1 modulates the activation and cleavage of key proteins involved in apoptotic signaling cascades. It influences apoptosis by cleaving substrates such as caspases, Bid, and Bax, thereby regulating cell fate decisions related to survival or death. This regulation is essential for proper development and tissue homeostasis. Furthermore, the cleavage of cytoskeletal and signaling proteins by calpain-1 contributes to the morphological changes and cell remodeling characteristic of apoptosis. Pyroptosis, on the other hand, is a highly inflammatory form of programmed cell death, often associated with infections and inflammatory states. Calpain-1 contributes to this process by cleaving cytoskeletal proteins such as vimentin, leading to the loss of structural integrity and mechanical resistance of the cell. This promotes cell rupture under mechanical stress and the release of cellular contents. Additionally, calpain-1 participates in the maturation and release of pro-inflammatory cytokines, particularly IL-1α, thereby enhancing the inflammatory response. Unlike IL-1β, IL-1α is active in both its precursor and mature forms, but its processing influences its activity and release. Given these insights, calpain-1 operates on two fronts: structurally, by facilitating cell rupture, and functionally, by activating cytokines such as IL-1α. Understanding calpain-1's role in these cell death processes is crucial for elucidating the mechanisms of programmed cell death and their significance in the pathogenesis of various diseases. I aimed to investigate these processes using selective chemical tools that allow for the detection of calpain-1 catalytic activity with high specificity and sensitivity.

To reach these goals, I focused on designing and synthesizing selective chemical tools to detect calpain-1, which enabled a more detailed understanding of its role in regulating cellular responses to stress and its potential as a therapeutic target in diseases linked to dysregulated cell death mechanisms. Studies on calpain-1 substrate specificity at the P4-P1 positions revealed its dual preference for hydrophobic amino acids (Phe, Tyr, Leu) and positively charged amino acids such as Arg at P1. At the P2 position, calpain shows a strong

preference for leucine, although other amino acids such as valine and isoleucine are also preferred. The remaining positions exhibit greater flexibility, allowing for the recognition of a wide range of amino acids. To investigate calpain-1's substrate preferences, I used IQF (internally-quenched fluorescence) peptide substrates, which enable the monitoring of protein cleavage through fluorescence emission. From the Hybrid Combinatorial Substrate Library (HyCoSuL) approach, I created optimal peptide sequences for detecting calpain-1 and synthesized a series of selective fluorescent substrates and inhibitors that distinguish calpain-1 from other proteases, such as proteasomes and cysteine cathepsins.

To enable the visualization of calpain-1 activity in cells, I developed activity-based fluorescent probes (ABP) specifically tailored to calpain-1 for the first time. This allowed me to monitor its activity in real-time in THP-1 cells treated with calcium ionophore or nigericin, providing valuable insights into the dynamics of calpain-1 activation in response to cellular stress. As the role of calpain-1 in apoptosis and pyroptosis is yet to be established, I sought to explore its potential role as a switch between these opposing cellular processes. Caspase-1 mediates pyroptosis by cleaving gasdermin D (GSDMD), leading to pore formation in the cell membrane and lytic cell death, whereas caspase-3 cleaves GSDMD fragments that lack pore-forming capability, promoting apoptosis. So I also investigated the involvement of calpain-1 in GSDMD cleavage and its role in regulating cell death. My studies showed that calpain-1 can indeed cleave GSDMD. To identify the cleavage sites, I developed fluorescently labeled peptide substrates based on GSDMD fragments. Finally, by using mass cytometry, I also analyzed the expression pattern of calpain-1 in immune cells at the single-cell level, revealing dominant expression of calpain-1 in granulocytes, co-localized with caspase-1 and gasdermin D. My findings suggest that calpain-1 plays a role in cell death processes, particularly in the context of neutrophils, which may open new avenues for research on this protease.