Rhomboid intramembrane serine proteases cleave polypeptide chains within lipid bilayer. Rhomboid proteases were originally discovered in *Drosophila melanogaster* where they regulate ontogenesis of the fly, but they are present in all domains of life. Nowadays, various diseases, such as malaria, amoebiasis, Parkinson's disease, various tumour malignancies, and diabetes, have been linked with rhomboid proteases. However, natural substrates and function of most rhomboids remain elusive. Cell biology tools are needed for unravelling functions of rhomboids, as well as for potential pharmacological applications, and this together fuels the effort to develop specific rhomboid inhibitors. The inhibitors known to date always bear an electrophilic warhead attacking the nucleophilic serine of the atypical serine-histidine catalytic dyad of rhomboid.

From the various developed inhibitors, peptidyl α-ketoamides substituted at the ketoamide nitrogen by hydrophobic groups, discovered in our laboratory, hold the biggest potential. They are potent, reversible, selective, tunable, and are built around a pharmacophore already approved for medical use. Here, I set out to improve peptidyl α-ketoamides by exploring the chemical space in the active site of rhomboid and testing substituents of the ketoamide nitrogen of increasing size, different structure and chemical nature. Branching of the hydrophobic substituents improved inhibitory potency. Of the inhibitors bearing branched substituents, compound **22** was the most potent one. It inhibited endogenous *E. coli* rhomboid GlpG, meaning that it was able to cross the bacterial outer membrane. To understand the reasons for its improved potency in the absence of a co-crystal structure, I analysed a computational model of the complex. It revealed that the branched substituent of compound **22** extensively occupies the prime side of the rhomboid active site forming numerous interactions with the fifth transmembrane helix and loop L5 of the rhomboid. These regions of rhomboid proteases are hypothesized to participate in substrate binding and cleavage. Since rhomboid protease architecture and mechanism is conserved, these findings give impetus to explore the strategy of using branched substituents of ketoamide nitrogen in designing inhibitors of other rhomboids for studying their biological roles and/or for their pharmacological targeting.