

Protease Site-directed Mutagenesis Distinguishes Cannibalistic Interactions in Proteolytic Networks: Confounded Pharmaceutical Inhibitor Kinetics

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Introduction: Cysteine cathepsin proteases include some of the most powerful human collagenases and elastases and are implicated in cardiovascular disease, where collagen and elastin are integral in artery biomechanics and signaling. Of particular interest are cathepsins K, L, and S, which share 60% sequence homology and redundancy in target substrate proteins with different catalytic activities toward different extracellular matrix substrates, which have been investigated in isolation. However, cells secrete many proteases simultaneously to remodel arterial wall during disease progression.

This has made them highly sought after pharmaceutical targets, but of the 16 well-designed inhibitors that have advanced to phase II clinical trials, all have failed except one due to cross-reactivity and off-target side effects, although there was efficacy for slowing disease progression. Previously, it has been shown that one species of cathepsin will preferentially degrade another, while also in the presence of matrix proteins; which we termed *cathepsin cannibalism*. This led to the hypothesis that off-target side effects of pharmaceutical cathepsin inhibitors may be due to an incomplete understanding of cathepsin regulation of each other, which could impact drug dosing. Here we use computational modeling to shed light on the proteolytic network of cathepsin cannibalism between multiple species and substrates to probe this network that is experimentally difficult to study to understand the kinetics of small molecule inhibitors for appropriate dosing for small molecule inhibition of these cathepsins. Furthermore, we made site-specific mutants to confirm cannibalism cleavage directionality.

Materials and Methods: Kinetic assays of fluorogenic substrates, which fluoresce when cleaved, with different combinations of recombinant cathepsins (cat) K, L, and S were performed to determine time series substrate degradation over 120 minutes. The model, developed using a general mass action model, fit the kinetic parameters of each cathepsins to time series data of substrate degradation of different combinations of cathepsins. Parameters of the mass action rates were determined from the kinetic assay using local optimization. To validate cannibalism directionality terms from the model, cathepsin mutants were made using site-directed mutagenesis to mutate potential putative cannibalistic cleavage sites. These mutants are currently being employed in kinetic studies with fluorescent substrates to confirm *a priori* predicted preferential direction of cannibalism.

Results and Discussion: Cathepsin cannibalism allowed prediction of substrate degradation between pairs of catK, L, and S on gelatin and elastin. Although cathepsins have different affinities towards collagen and elastin, we see that catS degrades catK and catL, while catK degrades catL, independent of substrate. Fig. 1 shows that when there is catL and twice as much catS, there is less elastin degradation compared to either enzyme alone. The red-outlined and blue-outlined-white circles highlight that there are more complex protease interactions than just cathepsins degrading elastin by pre-incubating catL or catS with E64, a small molecule inhibitor.

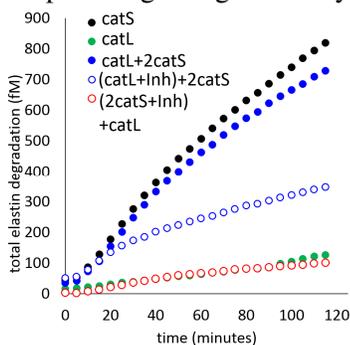


Figure 1. Total elastin degradation by different combinations of cathepsin L, S, and small molecule inhibitor, E64. The black circles and the green circles indicated elastin degradation by catS alone and catL alone, respectively. The blue circles indicate elastin degradation by when catL is combined with twice as much catS, however, there is less elastin degradation than either enzyme alone. The blue-and-white and red-and-white circles indicate elastin degradation when cathepsins are previously co-incubated with a small molecule inhibitor, E64. The blue-and-white circles essentially show the activity of catL and catS degrading elastin, which is far less than the catS alone (black circles) case, indicating more complex interactions are taking place between these cathepsins.

Conclusions: Here we have shown that multiple cathepsin species in a single system can protect and preserve gelatin and elastin matrices due to cathepsin cannibalism, as observed between catK, L, and S. Furthermore, we analyzed a specific case between catL, S, and E64, showing that there are more complex interactions between these proteases, other than them just degrading substrate, supporting the idea that understanding these relationships is important for correctly dosing small molecule inhibitors. Extending this further, we are also performing kinetic studies with multiple combinations of mutant and wild-type cathepsins to see the impact of these mutations on enzyme kinetics. It is imperative to understand consequences of having multiple proteases present in developing clinically relevant small molecule inhibition strategies for tissue destructive diseases.