Elucidating the Cathepsin Proteolytic Networks with Informed Mutagenesis and Purification Strategies

Marc A. Shuler, Meghan C. Ferrall, Maurizio Affer, Manu O. Platt
Wallace H. Coulter Department of Biomedical Engineering at Georgia Institute of Technology and Emory University, Atlanta, GA, USA

Introduction: Cysteine cathepsins are proteolytic enzymes that play an integral role in tissue destructive diseases such as atherosclerosis, cancer and osteoporosis by degrading extracellular matrix including collagen, gelatin and elastin. As such, pharmaceutical inhibitors have been developed to target them, however, all but one of the 16 drugs that has made it to phase II clinical trials has been discontinued due to side effects. There is a need to understand why these well-designed inhibitors are not working *in vivo*. Previous research has shown that some cathepsins will preferentially degrade other cathepsins, even in the presence of target substrate, a behavior known as *cathepsin cannibalism*. Determining cathepsin cannibalism directionality and substrate preferences will begin to unravel the complex proteolytic network dynamics of these enzymes, and provide insight into their levels *in vivo* that complicate pharmaceutical dosing and inhibition strategies.

Our hypothesis is that *specific sites on the cathepsin proteins are susceptible to cleavage by other cathepsins and mutagenesis at these probable sites will prevent cathepsin cannibalism and identify its preferred directionality.* To test this hypothesis, site-directed mutagenesis was used to introduce point mutations at locations deemed susceptible to cleavage using a bioinformatics approach. Cannibalism resistant and active-site dead mutants were created to determine direction of cannibalistic interactions. Then to verify the retention or loss of catalytic activity we conducted a multiplex cathepsin zymography and kinetic assays with fluorescent extracellular matrix substrates.

Materials and Methods: Cathepsin mutations were made and recombined into a pcDNA4/TO/myc-His vector that adds a C-terminal His tag to the protein being expressed for purification purposes. HEK293T cells were transformed for expression, and lysates were collected to purify recombinant mutant enzyme on nickel columns. To test for activity, cathepsin mutants were assayed via multiplex cathepsin zymography, a modified SDS-PAGE method to image active cathepsins after renaturation in polyacrylamide gels. In addition, kinetic assays of soluble purified cathepsins incubated with fluorescently quenched elastin in the presence or absence of the small molecule inhibitor E-64 was conducted to confirm elastinolytic activity of the mutant enzymes.

Results and Discussion: Active-site dead cathepsin K mutants (N296D, H276Q and C139S) demonstrated reduced ability to degrade gelatin in zymograms (Fig 1), and Western blot confirmed protein was still present, suggesting successful disruption of active-site catalysis. Cannibalism-resistant mutants (L253A, V171A and L253V) were capable of substrate degradation, as indicated by cleared white bands in the zymogram. Kinetic studies of elastin degradation by cathepsins S and V mutants indicated that they also remained active towards this matrix substrate. An average of 25% decrease in substrate degradation was observed in the presence of E-64 as an indicator of the cathepsin specific contribution.

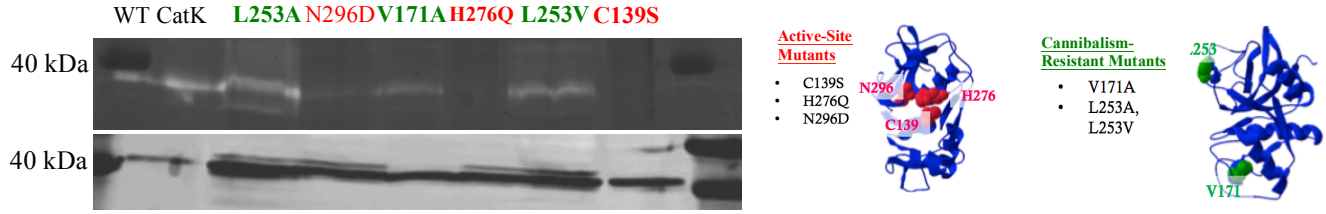

Figure 1: Cathepsin K wild type & mutants zymography (above) detect mutant activity and western blot (below) indicates enzymes are present. Active-site dead mutants (red) did not degrade gelatin, thus lack of white band. Cannibalism-resistant mutants (green) remained active, as expected. Western blot showed target protein is present in wild type and all mutants. Locations of these single point mutations are shown in the cathepsin K diagrams depicted.

Conclusions: Cannibalism-resistant cathepsin K mutants retained ability to degrade targets substrates. Active-site mutants showed little to no substrate degradation, suggesting successful disruption of active-site binding. Cathepsin S & V mutants showed activity and the ability to be inhibited by E-64. With purified mutants, we can determine cannibalism directionality between cathepsin K, S, and V by co-incubating pairs of wildtype and mutant cathepsins together and confirming an increase in total substrate degradation with cannibalism-resistant mutants and decrease with active-site mutants.