

Characterization of Elastolytic Cathepsins in Macrophages

by

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ABSTRACT

Atherosclerosis is characterized by a thickening of the arterial wall and loss of its elasticity. The elasticity of the arterial wall is impaired when the extracellular matrix undergoes extensive proteolytic remodeling. Cathepsins are papain-like cysteine proteases that are known to have elastolytic/fibrinolytic activities. They are highly expressed in macrophages present in plaque areas of diseased blood vessels and are thought to contribute to the tissue remodeling. Using cathepsin deficient macrophages and various protease inhibitors, the elastolytic activities of cathepsins B, K, L, and S were quantitatively determined. Up to 60% of the total elastase activity of macrophages was attributed to cathepsin activities. Deficiencies in single cathepsins appeared to be compensated by other cathepsins. The capability and potency of cathepsins B, K, L, and V to hydrolyze fibrin was also determined.

The exact quantification of individual cathepsin activities with the help of inhibitors or enzyme deficiencies in biological samples is difficult due to compensatory effects. Thus, specific substrates could be a viable alternative. Commercially available cathepsin activity assay kits that exploit fluorogenic peptidyl substrates are widely used to measure individual cathepsin activities in biological samples. However, substrates marketed as cathepsin K, L and S specific were found to be only marginally specific or completely non-specific, and were hydrolyzed by various other cathepsins. Furthermore, the presence of highly potent endogenous inhibitors in biological samples and the lack of specificity of the substrates skew the measurements towards cathepsin B which is relatively resistant to endogenous

inhibitors. Thus, data obtained using commercial substrate kits are to be interpreted with great caution.

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LIST OF ABBREVIATIONS

AC-FF-FMK	Acetyl-phenylalanine-phenylalanine-fluoromethyl ketone
AMC	7-Amino-4-methyl coumarin
CA074	L-trans-Epoxy succinyl- isoleucyl-proline OH propylamide
CA074-Ome	L-trans-Epoxy succinyl-isoleucyl-proline methyl ester propylamide
CatSI	Cathepsin S inhibitor
CVD	Cardiovascular disease
DAPI	4,6-diamidino-2-phenylindole
3,4-DCIC	3,4-Dichloroisocoumarin
DMEM	Dulbecco's modified eagle medium
DQ-elastin	Soluble bovine neck ligament elastin conjugated with BODIPY-FL
DTT	Dithiothreitol
E64	L-trans-Epoxy succinyl-leucylamido(4-guanidino)butane
E64d	L-trans-epoxy succinyl (OEt)-leu-3-methylbutylamide-ethyl ester
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EtBr	Ethidium bromide
FDP	Fibrin degradation product
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum

GM6001	N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide
HBS	Hepes buffered saline
K17	Morpholino-leucine-homophenylalanine-vinylsulfone
LDH	Lactate dehydrogenase
MCA	7-methyl-aminocoumarin amide
MGC	Multi-nucleated giant cell
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NaCl	Sodium chloride
NE	Neutrophil elastase
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RPMI	Roswell park memorial institute
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
TGC	Thioglycollate
Tris	(tris)Hydroxymethylaminomethane
Z-RR-MCA	Carbobenzoxy-arginine-arginine-4-methylcoumarin-7-amide
Z-LR-MCA	Carbobenzoxy-leucine-arginine-4-methylcoumarin-7-amide
Z-FR-MCA	Carbobenzoxy-phenylalanine-arginine-4-methylcoumarin-7-amide
Z-VVR-MCA	Carbobenzoxy-valine-valine-arginine-4-methylcoumarin-7-amide

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DEDICATION

This work is dedicated to B, S, & J

1INTRODUCTION

1.1 Proteases

A protease is an enzyme that hydrolyzes peptide bonds in a peptide chain. Proteases can be categorized into subgroups according to their substrate specificity and mechanisms of action. Proteases are classified by their sites of cleavages within a polypeptide chain or their catalytic mechanisms. For instance, proteases that cleave within polypeptides are endopeptidases, and those that cleave at the ends of polypeptides are called exopeptidases. Aminopeptidases and carboxypeptidases cleave at the N-terminal and C-terminal of polypeptides, respectively. Based on the mechanism of catalysis, proteases are divided into 5 groups, which include aspartic, cysteine, serine, threonine, and metalloproteinases. In the following, I will be mostly focusing on papain-like cysteine proteases, also known as cathepsin cysteine proteases.

Papain-like cysteine proteases are expressed in the animal/plant kingdoms as well as in bacteria and viruses. There are a total number of 11 cathepsins that belong to the papain superfamily, and they contain of cathepsins B, C, F, H, K, L, V, O, S, W and X. All papain-like cysteine proteases consist of a signal peptide, a pro-peptide, and a catalytic domain, which is known to be highly conserved among cysteine proteases [1].

Cathepsins	Signal peptide (AA)	Prodomain		Mature domain		Prepro-enzyme	
		Length (AA)	Size (Da)	Length (AA)	Size (Da)	Length (AA)	Size (Da)
Cathepsin B	17	62	7245	260	28663	339	37807
Cathepsin K	15	99	11833	215	23495	329	36966
Cathepsin L	17	96	11724	220	24170	333	37546
Cathepsin S	16	98	11830	217	23993	331	37479
Cathepsin V	17	96	11672	221	23999	334	37329

Table1.1 Human Cysteine Cathepsin Domain Organization Values taken from *Lecaille et al* 2008 [2].

The signal peptide is essential for proper translocation of the enzyme into the endoplasmic reticulum after translation, and they are usually 10-20 amino acid residues long (Table 1.1) [1]. The signal peptide is removed during the passage to the endoplasmic reticulum and pro-cathepsins are formed. The length of a pro-peptide is between 50-250 amino acid residues, and they play a crucial role before the enzymes become proteolytically active [1]. There are 3 major functions of the prodomains: i) they provide a stable scaffold for proper folding of the catalytic domain [3, 4], ii) they serve as chaperone when the proenzymes are being transported through the lysosomal/endosomal compartments [5, 6], and iii) they prevent premature activation by serving as an inhibitor [7-10]. Briefly, they are responsible for proper folding and stability at neutral pH. The catalytic domains of papain-like cathepsins are 220-260 amino acid residues long and they have highest conservation among the three polypeptide domains. All cysteine proteases have conserved active site residues which include Cysteine, Histidine, and Asparagine which are essential for the mechanism of catalytic activities.

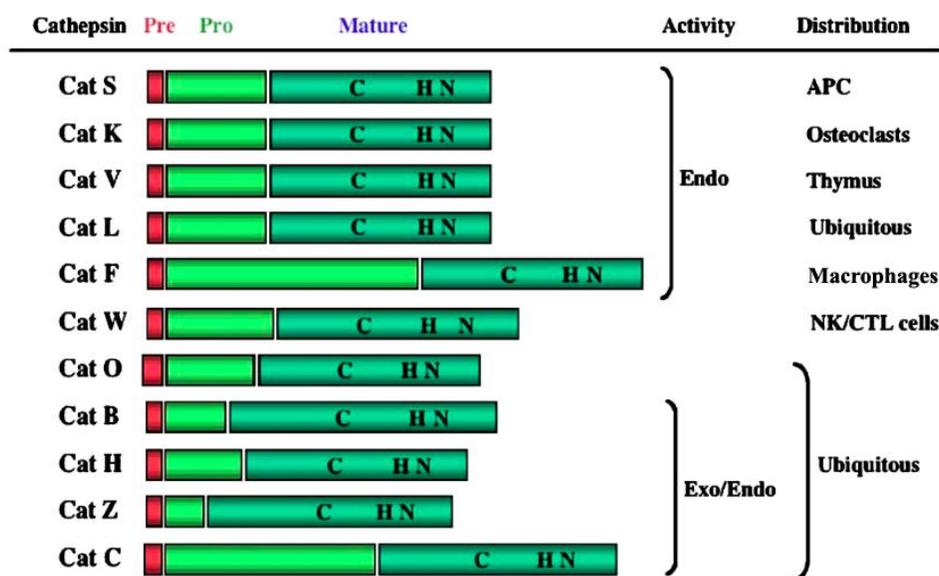


Figure 1.1 Human Papain-Like Cysteine Proteases. Picture was taken from *Yasuda et al* 2005 [11].

1.2 Catalytic Mechanism of Cathepsin Cysteine Proteases

Cathepsins perform peptide bond hydrolysis by utilizing a catalytic triad mechanism. The catalytic triad involves 3 residues in the active site: Cysteine²⁵, Histidine¹⁵⁹, and Asparagine¹⁷⁵ (according to papain numbering).

As shown in figure 1.2, the formation of thiolate-imidazolium ion pair is established by Cys²⁵ and His¹⁶², and it is stabilized by the hydrogen bond between Asn¹⁷⁵ and His¹⁶²[12]. The thiolate anion is considered as a powerful nucleophile which has a great ability of attacking the carbonyl carbon of the substrate. The cleavage of substrate bond leads to the formation of an unstable tetrahedral intermediate, which is stabilized by the oxyanion hole

[13, 14]. The intermediate is then transformed into an acyl enzyme when the protonated amine is released [2]. Then, the water molecule is able to attack the carbonyl carbon again in order to form second tetrahedral intermediate. Finally, the intermediate is deacylated, and the second portion of the substrate (R-COOH) is released[1]. The free enzyme is now available to hydrolyze brand new substrates.

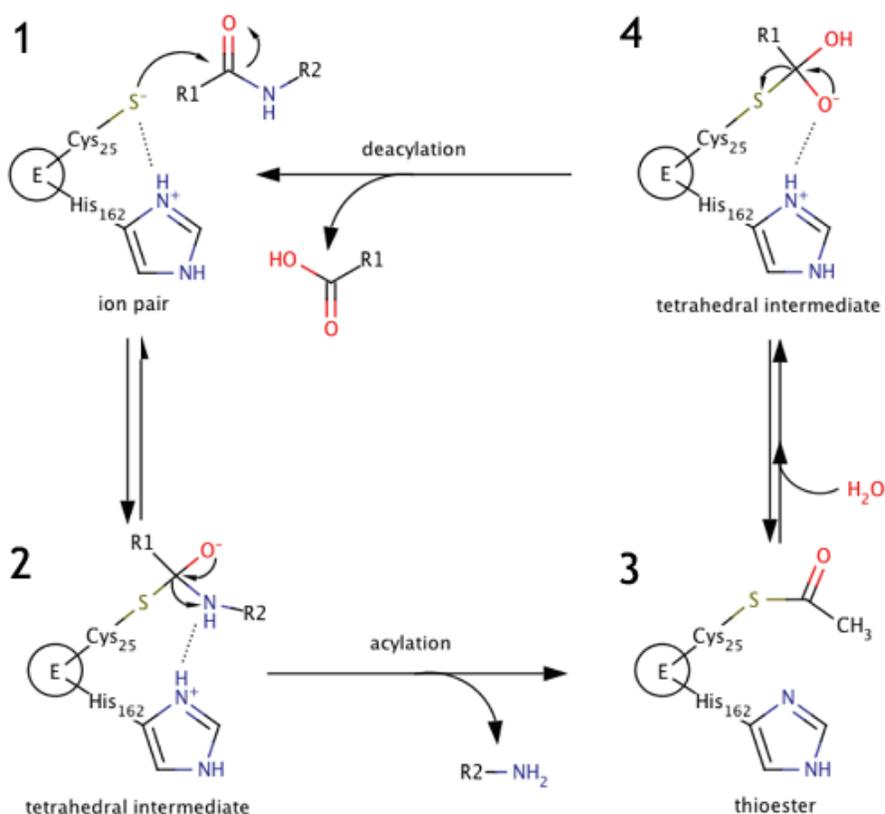


Figure 1.2 Mechanism of Substrate Hydrolysis by Cathepsin Cysteine Proteases.

The catalytic triad of cathepsins consists of Cys²⁵ and His¹⁶², and it is stabilized by the hydrogen bond between Asn¹⁷⁵ and His¹⁶². A thiolate anion attacks the substrate, which forms a tetrahedral intermediate. The peptide is cleaved by an acylation reaction forming a thiolester, and it reacts with water to produce another tetrahedral intermediate, which is then deacylated generating a free enzyme. Figure was modified from *Lecaille et al 2002* [1] and taken from Vincent Lavalée's thesis (2011).

1.3 Intracellular and Tissue Distributions of Cathepsin Cysteine Proteases

Cathepsins are synthesized in the rough endoplasmic reticulum as preproenzymes as described above. They undergo extensive processing such as N-linked glycosylation, which is necessary for translocation to the lysosomal compartment. Cathepsin cysteine proteases are not only localized to lysosomes, but they also reside in phagosomes, endosomes, and may accumulate in specific organelles under pathological conditions. For example, cathepsin H is highly expressed in the early endosomes of macrophages, whereas cathepsin S appears to be concentrated in the late endosomes [15]. Moreover, cathepsin W is usually found in the endoplasmic reticulum [16]. One of the reasons why cathepsins were thought to be only retained in the lysosomes is that cathepsins are optimally active at acidic pH with the exception of cathepsin S which retains its activity also at neutral pH [17-19].

Mammalian cathepsins are either ubiquitously or tissue and cell specifically expressed (figure 1.1) [19]. Furthermore, the expression levels of cathepsins are dramatically altered in pathological conditions such as in cardiovascular disease [20, 21]. Cathepsins are expressed throughout different types of tissues and cells, which suggest their housekeeping roles. For instance, cathepsins B, L, H, C, X, F, and O are considered to be expressed in many organs carrying out housekeeping functions. On the other hand, there are certain cathepsins that are expressed in a highly selective manner, which imply specific roles of certain cathepsins. As shown in figure 1.3, cathepsins are involved in a variety of physiological conditions. For example, cathepsin K is predominantly expressed in osteoclasts and multi-nucleated giant cells (MGCs), and plays a key role in bone

remodeling and extracellular matrix degradation [11, 22]. Moreover, cathepsin S is selectively expressed in antigen-presenting cells such as lymphatic tissues or splenocytes [23]. Cathepsins F, L, S, and V are known to be involved in major histocompatibility complex (MHC) class II antigen presentation and immune response [24]. Cathepsins B and L are known to play a crucial role in prohormone processing [25].

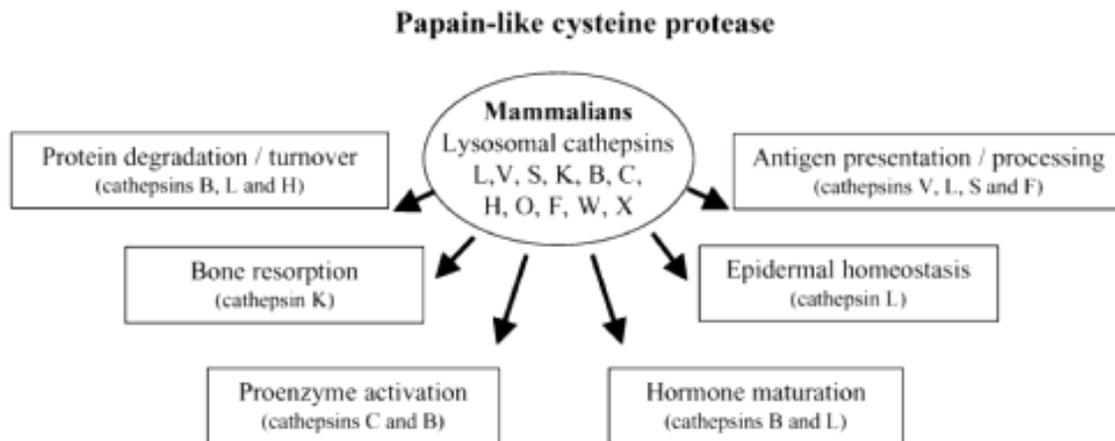


Figure 1.3 Physiological Role of Mammalian Cathepsin. Cathepsins B, L, H, C, X, and O are expressed ubiquitously and are involved in housekeeping functions. Other cathepsins are expressed in specific tissues implicating distinct roles in cellular functions. Figure was taken from *Lecaille et al* 2002 [1].

The involvement of cathepsins in homeostasis and protein turnover suggests that they are closely related to diseases, and contribute to pathological conditions. Furthermore, there are cathepsin-deficient mice models utilized in a variety of experiments that allows the investigation of cathepsin-related diseases. In the following, the association of cathepsins in the development of atherosclerosis will be discussed.

1.4 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death in developed countries [26]. Atherosclerosis is a form of CVD characterized by subsequent lipid deposition and continuous remodeling of extracellular matrix (ECM) of the blood vessel wall [27, 28].

Atherosclerosis can be divided into 4 stages [29]. The earliest stage of atherosclerosis is defined by increased endothelial permeability to lipoproteins and other plasma constituents, which eventually up-regulates leukocyte adhesion and migration. In the intermediate stage, fibrin present within the plaque stimulates the formation of the fatty streaks by recruiting platelets and leukocytes to the lesion. The proliferation and migration of smooth muscle cells are also promoted, which further contributes to the growth of the plaque. In the advanced stage, the formation of a necrotic core and a fibrous-cap occurs, and the fatty streaks progress to complex lesions (figure 1.4). The advanced plaque contains increased levels of fibrin and fibrin degradation products (FDP) within the fibrous-cap and necrotic core regions [30]. Accumulation of macrophage foam cells, fibrin deposition, and inflammatory cytokines promote further thickening and occlusion of the lesions. Finally, the degradation of the fibrous cap by proteases may cause rupturing of the plaque which may eventually cause thrombosis. Macrophages, smooth muscles cells, and endothelial cells express high levels of proteases that degrade the fibrous cap or the matrix.

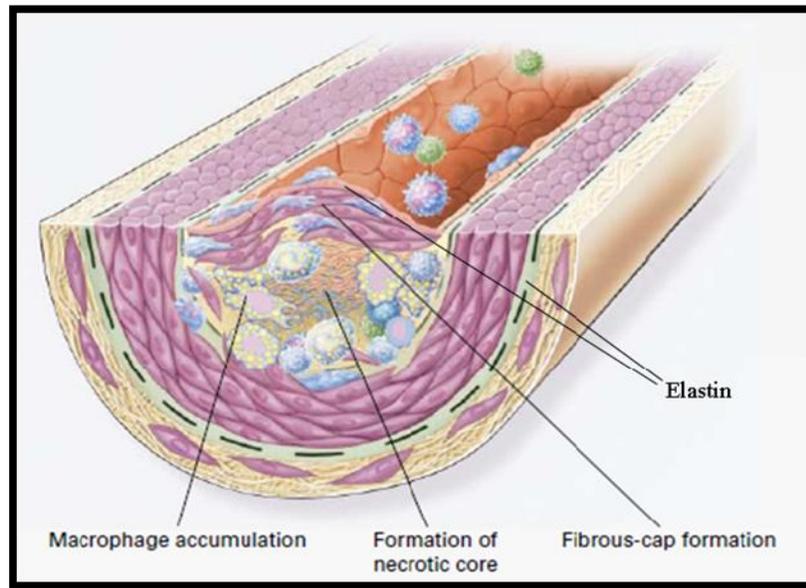


Figure 1.4 Formation of an Advanced Lesion in Atherosclerosis. Atherosclerosis is an inflammatory disease that includes extensive remodeling of the extracellular matrix due to increased expressions of proteases. Figure was taken from *Ross et al* 1999 [29].

Arterial ECM is composed of a variety of constituents such as elastin, collagen, and proteoglycans, which provide rigidity, elasticity, and permeability towards the arterial wall. Remodeling of the ECM exists in two types, where one is the physiological process of maintenance, and the other is pathological remodeling that may lead to fatal outcome. Proteolytic enzymes play an important role in remodeling of the ECM architecture of the arterial wall and pathological development.

There are major arterial proteases that are closely involved in the pathophysiological process of atherosclerosis. In atherosclerotic lesions, the importance of matrix metalloproteinases (MMPs) and serine proteases has been well established [31-33]. Macrophages and SMCs in the regions of well-developed lesions and sites prone to rupture

are known to express increased levels of MMP-1, -2, -3, -7, -8, -9, -13, and metalloelastase-12 (MMP-12) [34-37]. Previous findings showed that neutrophil elastase (NE) was highly expressed in atherosclerotic lesions and played an important role in matrix breakdown [38]. Furthermore, serine proteases were found to be involved in the regulation of MMP expressions from SMCs, which degrade matrix proteins within the atherosclerotic lesions [39, 40]. Despite of these important proteases in ECM catabolism, there are other proteases such as cathepsins that have been found to play equally important roles in ECM degradation [41].

It was shown that macrophages or lipid-rich areas express high levels of active cathepsins including cathepsins B, K, L, and S [42-44], which contribute to extensive ECM remodeling. For example, cathepsin S deficiency reduced the atherosclerotic plaque size by 46% and reduced the macrophage and leukocyte accumulation in atherosclerotic lesions [45]. Furthermore, deficiency in cathepsin K expression reduced plaque progression and induced plaque fibrosis [44, 46]. It was found that cathepsins are closely related to the progressions of atherosclerosis, and ECM degradation, but the direct mechanism of how cathepsins degrade these proteins still remain uncertain. Cathepsins are known to be intracellular proteases, but they are also able to degrade extracellular elastin and collagen as well. It was found that monocyte-derived macrophages are able to express vacuolar-type H⁺-ATPase components, which acidifies the pericellular milieu, and thus provides the optimal environment for the activity of secreted, extracellular cathepsins [47]. Major extracellular proteins present in plaques are collagen, elastin and fibrin [30, 48, 49]. Collagen and elastin form the major structural components of the blood vessel wall and

established lesion within the diseased vessel. Plaques also contain significant amounts of fibrin which is thought to be derived from vascular microdamages [30]. All three components are degraded during the disease development by various cathepsins as has been shown in atherosclerotic mouse models [50, 51]. Here, I will focus on the degradation of elastin and fibrin. Figure 1.5 summarizes the expression pattern of cathepsins and the presence of extracellular matrix components in atherosclerotic plaques.

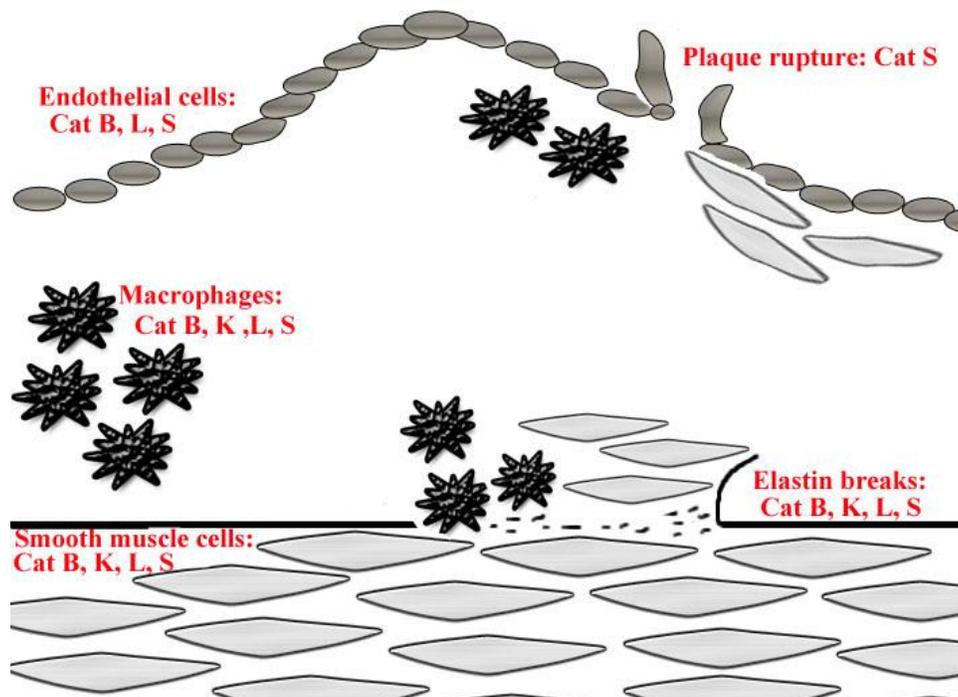


Figure 1.5 Overview of Cathepsin Expression and Activity in an Atherosclerotic Plaque. The expression of cathepsins K, L, and S is up-regulated in macrophages, SMCs (smooth muscle cells), and ECs (endothelial cells). The ECM is composed of elastin, collagen, and proteoglycans which undergo extensive remodeling due to increased cathepsin activities. Figure was modified after *Lutgens et al* 2007 [41].

1.5 Inhibitors of Cathepsin Cysteine Proteases

1.5.1 Natural Inhibitors of Cathepsin Cysteine Proteases

Cystatins are natural inhibitor for cathepsins, which form a large family. All cystatins consist of three conserved regions: i) N-terminal glycine-containing region, ii) central pentamer, and iii) conserved Pro¹⁰⁵-Trp¹⁰⁶ pair [2]. Cystatins are defined as reversible inhibitors that tightly bind to cathepsins in a competitive manner, and they are non-glycosylated single chain proteins with molecular masses of 13-14 kDa [2]. Cystatins are ubiquitously expressed in all body fluids, and cystatin C is one of them that has been widely studied for being one of the tightest-binding inhibitor of cysteine proteases [52, 53]. Cystatin C is capable of inhibiting cathepsin activities in the picomolar range.

Enzyme	Ki
Cathepsin B	250 pM
Cathepsin K	4.1 pM
Cathepsin L	9.5 pM
Cathepsin S	8.0 pM
Cathepsin V	20 pM

Table 1.2 Cathepsin Cysteine Proteases and Ki Values towards Cystatin C.

Cystatin C is known as an endogenous inhibitor for cathepsins, and they are inhibited at the picomolar range. Values were taken from *Bromme et al* 1991 [54, 55].

As mentioned previously, atherosclerotic lesions express high levels of cathepsins that contribute to abnormal remodeling of arterial wall. Interestingly, it has been suggested that

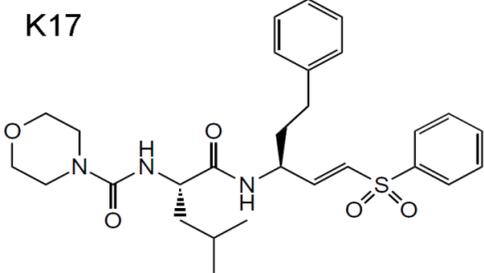
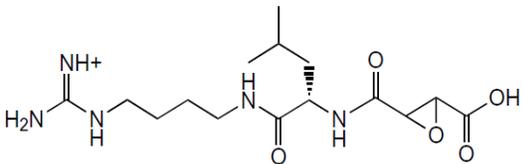
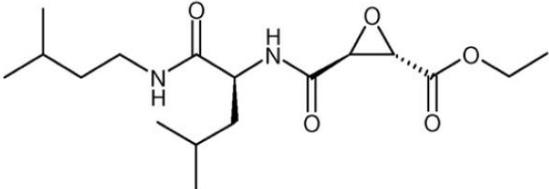
there are also severely reduced levels of cystatin C in both atherosclerotic and aneurysmal aortic lesion [56]. Cystatin C deficiency increased the degradation of elastic lamina and decreased the medial size [57]. Moreover, cystatin C deficient mice were fed with high fat diet for 25 weeks using the apoE-deficient atherosclerotic model. Mice revealed a significant increase in plaque size, as well as increased lipid content and macrophage accumulations at the site of plaque progression [58]. Properties of cystatins and other endogenous inhibitors of cathepsins possess critical information for developing synthetic inhibitors to treat cathepsin-related diseases.

1.5.2 Synthetic Inhibitors of Cathepsins and other Proteases

The design and synthesis of protease inhibitors has received great attention over the past decade, and they have been extensively reviewed in recent years. [1, 59]. Protease inhibitors are of great pharmacological value as they can be used as potential drugs for specific diseases. In this study, six cysteine protease inhibitors and two non-cysteine protease inhibitors were utilized to investigate the contributions of proteases to elastin degradation in macrophages. The name, structure and function of these inhibitors are shown in Table 1.3.

K17 is an irreversible vinyl sulfone inhibitor of cysteine proteases, and it is known to completely inhibit extra and intracellular cathepsin activities at a concentration of 10 μ M. E64 (extracellular) and E64d (intracellular) are epoxysuccinyl-based cathepsin cysteine protease inhibitors which were used to characterize extracellular and intracellular

degradation of elastin by cathepsins. CatSI is a nitrile-based inhibitor for cathepsin S, which is highly potent and selective towards cathepsin S. CA074 (extracellular) and CA074-Ome (intracellular) are irreversible and selective inhibitors of cathepsin B. CA074-Ome is the methylated version of CA074, and it is able to move across the cell membrane inhibiting intracellular cathepsin B activity. 3,4-DCIC, also known as 3,4-dichloroisocoumarin, is a general inhibitor of serine proteases. GM6001 is a reversible matrix metalloproteinase (MMP) inhibitor which is a member of hydroxamic acid class.

Name (Assay concentration)	Function	Structure
K17 (10 μ M)	Extracellular and intracellular cathepsin inhibitor	<p>K17</p> 
E64 (10 μ M)	Extracellular cathepsin inhibitor	<p>E-64</p> 
E64d (10 μ M)	Intracellular cathepsin inhibitor	<p>E-64d</p> 

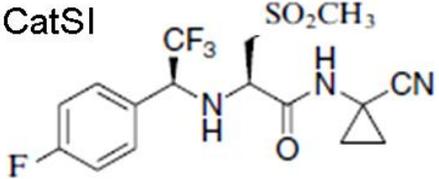
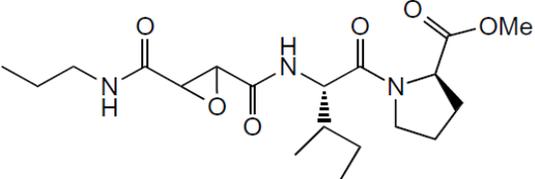
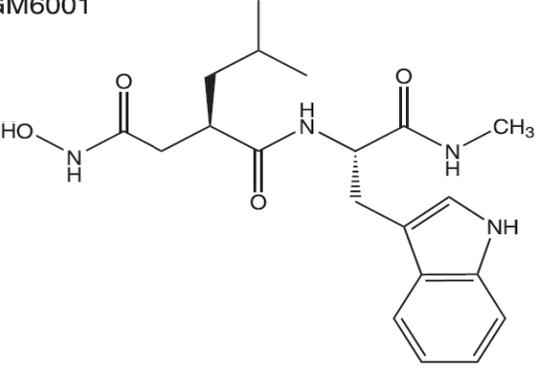
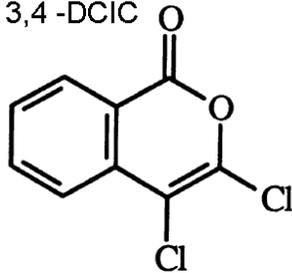
Name (Assay concentration)	Function	Structure
CatSI (500 nM)	Extra and intracellular cathepsin S inhibitor	<p>CatSI</p> 
CA074-Ome (10 μM)	Intracellular cathepsin B inhibitor	<p>CA-074-OMe</p> 
GM6001 (10 μM)	MMP inhibitor	<p>GM6001</p> 
3,4 DCIC (10 μM)	Serine protease inhibitor	<p>3,4 -DCIC</p> 

Table 1.3 The Name, Structure, and Functions of Inhibitors of Cathepsin Cysteine Protease, Matrix Metalloproteinase, and Serine Protease. [60-63]

1.6 Synthetic and Semi-synthetic Substrates for Cathepsin Cysteine Proteases

There are many synthetic substrates that are used in order to measure enzymatic activities of cathepsin cysteine proteases [64]. There are four widely used fluorogenic substrates for cathepsins K, L, S, And B: i) Carbobenzoxy-arginine-arginine-methyl-7-aminocoumarin amide (Z-RR-MCA), ii) Carbobenzoxy-leucine-arginine-methyl-7-aminocoumarin amide (Z-LR-MCA), iii) Carbobenzoxy-phenylalanine-arginine-methyl-7-aminocoumarin amide (Z-FR-MCA), and iv) Carbobenzoxy-valine-valine-arginine-methyl-7-aminocoumarin amide (Z-VVR-MCA).

The fluorescence properties of the fluorescent tag, MCA, also known as methyl-7-aminocoumarin amide, are altered upon cleavage of the amide bond. Released 7-amino-4-methyl coumarin (AMC) is highly fluorescent and is recorded in a fluorometer. The fluorogenic substrate binds to the active site of cathepsins and occupies both the prime and non-prime regions with the MCA group placed in the S'₁ subsite of cathepsins [65]. The X group (figure 1.6) is a representative of an amino acid or peptide and binds on the non-prime site.

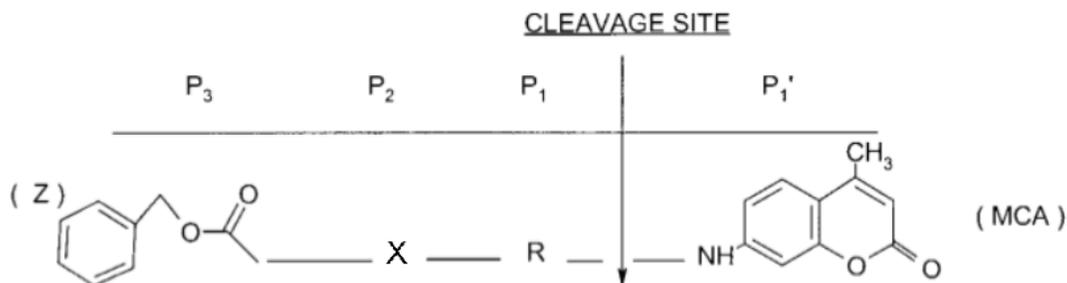


Figure 1.6 Z-XR-MCA Cleavage Site by Cathepsin Cysteine Proteases. Z-XR-MCA is cleaved by cathepsins with the arginine group bound to the P₁ site and the MCA group bound to the P₁' site adjacent to the arginine. The hydrolysis converts the MCA group into AMC molecule providing quantitative fluorescent signals. X = any amino acid such as R, L, F, V [64].

There are also other semi-synthetic substrates, which includes fluorescein-elastin, congo-red elastin, and DQ-elastin. These substrates are widely used to quantify the elastolytic activities of cathepsins and other elastases [66, 67]. Fluorescein-elastin is generated by labeling the bovine neck ligament with fluorescein-isothiocyanate. The insoluble fluorescein-elastin becomes soluble when digested by elastases, and the fluorescent intensity can be measured with the excitation and emission wavelengths set at 490 nm and 520 nm, respectively. Similarly, congo-red elastin is prepared by the method of Hall [68]; the bovine neck ligament is dyed with congo-red, and the release of congo-red dye upon cleavage (Absorbance = 485 nm) allows the determination of elastolytic activity. DQ-elastin is a soluble substrate for elastases that are labeled with BODIPY-FL dye such that the conjugate's fluorescence is quenched. The non-fluorescent DQ-elastin becomes fluorescent upon proteolytic cleavage and the digested products can be measured at excitation and emission wavelengths of 505 nm and 515 nm, respectively.

1.7 Research Hypothesis and Rationale

1.7.1 Objective 1: Quantification of Elastolytic Activities of Cathepsins B, K, L, and S Expressed in Mouse Peritoneal Macrophages and Quantification of Cathepsin-Mediated Fibrinolysis.

1.7.1.1 Hypothesis and Goals

Cathepsins are highly expressed in macrophages, especially under pathological conditions. Moreover, cathepsin expressing macrophages are highly present at atherosclerotic plaques that give rise to ECM breakdown and add complications to atherosclerosis. Macrophages are also found next to fibrin clots either inside of plaques or adjacent to extravasated microclots. My hypothesis is that cathepsins play a significant and enzyme-specific role in macrophage-mediated elastin and fibrin degradation. My goal is to quantify the contributions of individual macrophage-expressed cathepsins, including cathepsins B, K, L, and S towards elastin and fibrin degradation.

1.7.1.2 Experimental Approach

To test this hypothesis, transgenic mice, cell lines, and various inhibitors were utilized. Peritoneal macrophages were isolated from wild-type, cathepsin K^{-/-} and cathepsin L^{-/-} mice and they were treated with various protease inhibitors such as K17, E64, E64d, CatSI, CA074, CA074-ome, 3,4-DCIC, and GM6001. The use of wild-type, cathepsin K^{-/-} and

cathepsin L^{-/-} mice allowed me to compare and contrast the elastolytic activities of individual cathepsins expressed in macrophages. As substrate, soluble DQ-elastin was used which allowed the fluorogenic measurement of elastinolysis. For comparison, an immortal macrophage line, Raw 264.7, was utilized. Recombinant cathepsins and plasmin were used to measure the hydrolysis of fibrin clots.

1.7.2 Objective 2: Characterizing Fluorogenic Substrates to Quantify Individual Cathepsins

1.7.2.1 Hypothesis and Goals

Quantifying individual cathepsin activity is difficult due to the lack of cathepsin-specific substrates. My first research aim focused on the determination of the contribution of individual cathepsins to the overall elastinolysis catalyzed by macrophages. Cathepsin deficient mice and inhibitors were used to distinguish between activities of each cathepsin. However, my studies revealed that cathepsin deficiencies and potentially chronic chemical inhibition may lead to the up-regulation of other proteases and thus may obscure the contribution of individual cathepsins. Based on the available literature, commercially available cathepsin activity assay kits may allow the quantification of each cathepsin activity using fluorogenic substrates to overcome this hurdle. My goal was to evaluate those cathepsin activity assay kits for their specificity.

1.7.2.2 Experimental Approach

4 different peptidyl substrates were compared which are commercially offered to quantify the activities of cathepsins B, L, K, and S. Recombinant human cathepsins B, K, L, S, and V were used as proteases. Cell extracts and blood plasma/serum were collected in order to determine cathepsin activities within biological samples. Furthermore, I have purchased a cathepsin L activity assay kit in order to validate or invalidate the suitability of commercial cathepsin activity kits to measure specific cysteine proteases in biological samples.

2 MATERIALS AND METHODS

2.1 Elastolytic Activities of Cathepsins in Macrophages

2.1.1 Genotyping of Cathepsin K^{-/-} Mice and Cathepsin L^{-/-} Mice

Before starting any experiments with cathepsin deficient mice, genotyping was performed in order to confirm the cathepsin deficiency. Briefly, ear notches were collected from mice, and they were placed in Eppendorf tubes. 300 μ L of lysis buffer (100 mM NaCl (Fisher, Fair Lawn, New Jersey), 100 mM Tris (Fisher, Fair Lawn, New Jersey) pH8, 25 mM EDTA, 0.5% SDS) and 2 μ L of protease K (20 mg/mL) (Sigma, St. Louis, MO) were added to the ear notch samples. The samples were incubated at 37°C overnight. The next day, 100 μ L of protein precipitation solution (4 M guanidine thiocyanate, 0.1 M Tris, pH 7.5) were added to the samples and vortexed for 10 seconds. The samples were centrifuged at 15000 rpm for 5 minutes, and the supernatant was poured into a clean tube containing 300 μ L of 100% isopropanol. The tubes were mixed gently until the DNA pellet was visible to a naked eye. Once the pellet is formed, the samples were centrifuged at 15000 rpm for 1 minutes and the supernatant was discarded. 70% ethanol was added, mixed gently, and centrifuged at 15000 rpm for 1 minute. Once the 70% ethanol was discarded, the Eppendorf tubes were kept upside down for 10 minutes to get rid of the ethanol. 100 μ L of water was added for DNA hydration, and incubated at 65°C for 30 minutes. The tubes were tapped periodically to aid in dispersing DNA, and the samples were kept at 4°C for later use.

With these DNA samples, PCR reaction was performed for genotyping using the following primer pairs.

Cathepsin K	Primer Sequence
Cat K sense	5' - GCC ACA CCC ACA CCC TAG AAG- 3'
Cat K anti-sense	5' - ACA AGT GTA CAT TCC CGT ACC - 3'
Cathepsin L	Primer Sequence
MCL5	GGA GGA GAG CGA TAT GGG
MCL9a	TTC CTC ATT GGT CTT CCG
Neo756-777	CGG AGA ACC TGC GTG CAA TCC

The concentration of DNA samples was determined using Nanodrop 1000&8000 (Fisher, Wilmington, Delaware machine) and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the DNA ($A_{260}/A_{280} = \sim 1.8$). For each PCR reaction, 2.5 μ L 10x thermo buffer, 0.5 μ L 10 mM dNTP mix, 1 μ L 10 μ M primer mix, 0.2 μ L taq polymerase, 100 ng DNA was added, and the total sample volume was adjusted to 25 μ L with water. PCR conditions were as followed: 95°C for 1 minute, 25 repeats of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute. PCR product samples were analyzed by agarose gel electrophoresis. 2% agarose gel was made by adding 1g agarose (Sigma, St.Louis, MO) to 75 mL TAE buffer. The TAE buffer was prepared as a 50X stock solution, which contained 242g Tris in 500 mL H₂O, 100 mL 0.5M Na₂EDTA (pH 8), 57.1 mL glacial acetic acid, and adjusted volume to 1 L with H₂O. The agarose was dissolved in TAE buffer using a microwave, and the buffer was boiled until the volume reached 50 mL. It was poured to a gel cast, and kept at room temperature for 30 minutes for the process of polymerization to occur. While the agarose gel was being polymerized, 2.5 μ L agarose gel loading dye was added to the PCR product samples. After the polymerization of the agarose gel has occurred, the gel was taken out of the gel cast and placed into an agarose gel

running system. The PCR product samples were loaded as well as the 1Kb plus DNA ladder (Invitrogen, Burlington, ON) to the wells in the agarose gel. The agarose gel was run at 100 mA for 2 hours to confirm specific amplification. The gel was stained by ethidium bromide for DNA detection, and visualized using a bio imaging system (Syngene, Frederick, USA) under ultra violet light.

2.1.2 Generation of Primary Macrophages from Mice

Macrophages were generated using the standard thioglycollate (TGC) method. Before the injection of TGC, mice were placed into the induction chamber of the anesthetic machine. The oxygen flow meter was set at 0.8-1 L/min, the isoflurane vaporizer was adjusted to 2 - 3%, and the mice were anesthetized for 3 minutes. Once they were anesthetized, mice received 2 ml of 4% Brewer thioglycollate medium (Sigma, St. Louis, MO) by intraperitoneal injection using a 3 mL syringe (BD, Franklin Lakes, NJ) and a 26G3/8 needle (BD, Franklin Lakes, NJ). The mice were monitored twice a day to assess their health status. Mice were scored according to their body conditions from 0-6, where 0 being no signs of pain and discomfort and 6 being obvious signs of extreme pain, which requires immediate euthanasia. In the case of abdominal pain in these mice, anti-pain treatment took place using 0.05 mg/kg of buprenorphine. 4 days after injection, mice were anesthetized and euthanized by cervical dislocation for macrophage isolation.

2.1.3 Isolation of Murine Primary Macrophages

Macrophages were collected by peritoneal lavage with 8 mL of ice-cold RPMI 1640 medium. The skin of a mouse was cut open to reveal the peritoneal cavity. Using a syringe with a needle, 8 mL of RPMI (Sigma, St. Louis, MO) was injected into the peritoneal fluid. The peritoneal cavity received a gentle massage, and the fluid was collected using 9" cotton plugged Pasteur pipettes (Fisher, Burlington, Ontario). Macrophages were centrifuged at 1300 rpm for 4 minutes, and the RPMI 1640 medium was removed. Then macrophages were resuspended in 1mL of ice cold red blood cell lysis buffer (Sigma, St. Louis, MO) and incubated on ice for 5 minutes to lyse the red blood cells. Cells were spun down, and washed with ice cold phosphate buffered saline (PBS) (Sigma, St. Louis, MO) twice. After the washing step, cells were resuspended in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) (Sigma, St. Louis, MO), 100 U/mL penicillin (Gibco, Grand island, NY), 100 µg/mL streptomycin (Gibco, Grand island, NY), and 200 mM L-glutamine (Gibco, Grand island, NY). Cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA).

2.1.4 Macrophage Cell Line

The mouse macrophage cell line, Raw 264.7 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in a T75 flasks with DMEM supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 mM L-glutamine under 5% CO₂ at 37°C. Cells were cultured for 2-3 days until confluent,

and transferred to a T175 flask. Cells were detached from the flask using cell scraper and counted with a hemocytometer. Cells were ready to be used for the DQ elastin degradation assay.

2.1.5 Cell Viability Assay

To ensure the viability of the macrophage preparation under the assay conditions, the LDH cytotoxicity assay kit was used (Biovision, Mountain View, CA). Raw 264.7 macrophage cells were plated onto a 24-well plate with DMEM supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 200 mM L-glutamine under 5% CO₂ at 37°C. Cells were also treated with inhibitors including 10 µM K17 and 500 nM CatSI for 48 hours. The supernatant was collected and centrifuges at 250g for 10 minutes. 100 µL of the supernatant were transferred to an optically clear 96-well plate. 100 µL of reaction mixture was added to each well and incubated at room temperature for 30 minutes. The samples were protected from light. The absorbance was measure at 490-500 nm using a microtiter plate reader.

2.1.6 DQ-Elastin Degradation Assay and Inhibition Assays

The elastolytic activities expressed in macrophages were measured using soluble DQ-elastin from EnzChek Elastase Assay Kit (Molecular Probes/Invitrogen, Eugene, OR), and they were treated with protease inhibitors in order to characterize the contribution of cathepsins towards elastin degradation. DQ-elastin is derived from bovine neck elastic

ligaments and is fluorescently labeled with a proprietary dye, BODIPY-FL. 500,000 cells (either primary mouse macrophages or Raw 264.7) were plated onto a 24-well plate, and it was incubated for 2 hours to allow macrophages to adhere to the plate. The non-adherent cells were removed by washing with PBS. 700 μ L of serum-free RPMI 1640 medium containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 200 mM L-glutamine was added into each well. Cells were incubated for 24 hours with individually either 10 μ M K17, 10 μ M E64, 10 μ M E64d, 10 μ M CA074, 10 μ M CA074-Ome, 10 μ M GM6001, 10 μ M 3,4 DCIC, 500 nM CatSI (a selective cathepsin S inhibitor kindly provided by Merck-Frosst), or cocktails of those inhibitors [59]. After pre-incubation with inhibitors, the existing medium was removed, and changed to fresh serum-free RPMI medium containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 200 mM L-glutamine, 200 μ g DQ-elastin, and each designated inhibitors. The 24-well plate was incubated for another 24 hours. The supernatant were collected into 1.5 mL Eppendorf tubes and centrifuged at 1300 rpm. The fluorescence of the samples was determined at excitation 485 nm and emission 530 nm using Perkin Elmer luminescence spectrophotometer LS50B (Molecular Device, Sunnyvale, California).

The results were represented as mean \pm SD in percentage fluorescence from three independent experiments.

2.1.7 Intracellular Degradation of DQ-elastin Imaging

The intracellular elastolytic activity of macrophages was visualized using DQ-elastin from EnzChek Elastase Assay Kit. Raw 264.7 cell line was cultured in DMEM supplemented

with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 200mM L-glutamine under 5% CO₂ at 37°C. Then, macrophages were plated onto a 24-well plate with 200 µg DQ-elastin and inhibitors including 10 µM E64, 10 µM E64d, 10 µM CA074, and 10 µM CA074-Ome (all inhibitors were purchased from Sigma). After 24 hour incubation, cells were washed and mounted using DAPI for staining of the nuclei (SlowFade Gold, Invitrogen, Eugene, OR) and cells were observed using a Leica Fluorescence microscope at 40x magnification (Leica, Richmond Hill, Ontario).

2.1.8 Fibrinolysis Assay

The ability of cathepsins to degrade fibrin was tested and their efficiency was compared among cathepsins B, K, L, V, and plasmin. All human cathepsins were produced in *Pichia pastoris* and purified to homogeneity by chromatography [69] and were available to the laboratory. The fibrin clot was made using purified fibrinogen (3 µM), purified thrombin (3 nM), and CaCl₂ (5 mM) in hepes buffered saline-PEG (HBSPEG). The clot formation was performed in Costar 96 well flat bottom microplates, which were covered with clear sealer to prevent evaporation. 5 µL of thrombin, CaCl₂, and HBSPEG mixture was placed in each wells of the microplate. 95 µL of the fibrinogen and HBSPEG mixture were then added in order to initiate clotting. The plate was incubated at room temperature for 30 minutes to allow complete clot formation. While the clot is forming, 400 nM cathepsins B, K, L, V or plasmin were prepared in 100 mM sodium acetate buffer (pH 5.2) containing 2.5 mM DTT and 2.5 mM EDTA. 100 µL of each protease were placed on top of the fibrin clot, and it was ready for the fibrinolysis reaction. The micro plate reader (SpectraMax190, Molecular

Devices, Sunnyvale, California) was used in order to measure the turbidity of the clot at 405 nm for 10 hours at 27°C.

2.1.9 Statistics

DQ-elastin assay were done in triplicates and repeated three times. The fibrinolysis assay was done in duplicates and repeated twice. All data are expressed as the mean \pm standard deviation (SD). When applicable, the Student's unpaired t-test was performed. A probability level of < 0.05 signified a statistically significant difference. All statistics were evaluated using GraphPad Prism, version 5.0 for Mac OS X (GraphPad Software, San Diego, CA).

2.2 Characterization of Fluorogenic Substrates

2.2.1 Cathepsin Activity Assay

Activities of cathepsins B, K, L, S, and V were measured by the Barrett's method with some modifications [19], using 4 different fluorogenic substrates. The fluorogenic substrates used were Z-RR-MCA to measure cathepsin B, Z-LR-MCA to measure cathepsin K, Z-FR-MCA to measure cathepsins L and V, and Z-VVR-MCA to measure cathepsin S. All fluorogenic substrates were purchased from Bachem, Inc, King of Prussia, PA. We diluted 5 μ L of recombinant cathepsins (1 μ M stock) with 990 μ L using the reaction buffer

containing 100 mM sodium acetate (Fisher, Fair Lawn, New Jersey), 2.5 mM EDTA (pH 5.5) (Aeros, New Jersey, USA), and 2.5 mM DTT, at 37°C for 10 minutes. The assay was started by adding 5 μ L of substrate (5 μ M final), and the slope of the fluorogenic signal was recorded for 60 seconds. The enzymatic activity was measured by investigating the release of the fluorogenic leaving group at excitation and emission wavelengths of 380 nm and 450 nm, respectively, using a Perkin Elmer luminescence spectrophotometer LS50B. The enzymatic activity was followed by monitoring the release of the fluorogenic leaving group, MCA, at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using the Molecular Devices SpectraMax Gemini spectromicrofluorimeter.

2.2.2 High Substrate Concentration Cathepsin Activity Assay

The activities of cathepsins B, K, L, S, and V were measured by the same method as described above, except that the concentration of the 4 substrates was elevated to 200 μ M (final), and the fluorogenic signal was measured in the same manner as described above.

2.2.3 Measurement of Cathepsin Activities within Cell Homogenates

Raw264.7 macrophage cells were grown until confluence in a T175 flask, and cells were washed with ice-cold PBS twice. Cell were lysed using lysis buffer (pH 5.5) containing 20 mM sodium acetate, 150 mM sodium chloride (Fisher, Fair Lawn, New Jersey) , 1 mM EDTA , 1mM EGTA, 5 mM sodium fluoride (Sigma, Steinheim, Germany), 1% TritonX100 (Sigma, Steinheim, Germany), and 1 mM sodium orthovanadate. The cells were incubated

with lysis buffer for 60 minutes at 4°C, vortexing every 10 minutes. The cells were scraped from the flasks with a cell scraper, and the homogenates were collected. The homogenates were centrifuged for 5 minutes at 10,000 g at 4°C, and the supernatant was diluted with reaction buffer for the activity assay. For activity measurements, I diluted 20 µL of cell extracts to 200 µL using reaction buffer containing 100 mM sodium acetate, 2.5 mM EDTA (pH 5.5), and 2.5 mM DTT, at room temperature for 10 minutes. Cells were placed onto a 96 well plate, and different types of inhibitors were introduced. The assay was started by adding one of the 4 different substrates (5 µM), and the fluorogenic signal was monitored for an hour. The fluorescence excitation and emission was measured at 380 nm, and 450 nm respectively.

2.2.4 Measurement of Cathepsin Activities within Plasma/Serum (pH 5.5)

Activities of cathepsins were measure by the method described above. The fluorogenic peptide substrate used to measure cathepsin activity was Z-FR-MCA (5 µM). We diluted serial volumes of plasma/serum with reaction buffer (pH 5.5) and pre-incubated at room temperature for 10 minutes to obtain optimal activities of cathepsins expressed within the plasma/serum. The assay was started upon the addition of 5 µL substrate and the fluorogenic signal was measured at 380 nm and 450 nm, excitation and emission respectively, for 90 seconds using a Perkin Elmer luminescence spectrophotometer LS50B. In order to measure cathepsin S activity in plasma/serum, identical procedures were carried out except the acetate buffer was adjusted to pH 7 using TRIS base.

2.2.5 Measurement of the Stability of Cathepsin Activities within Plasma and Serum

The hydrolysis of fluorogenic substrate, Z-FR-MCA, was measured in plasma and serum samples with the addition of recombinant cathepsins B, K, L, S, and V. We combined 10 μL of plasma/serum with 10 μL of recombinant cathepsins, and 5 μL of the mixture was diluted to 995 μL of reaction buffer (pH 5.5). The assay was started by adding 5 μL of Z-FR-MCA (5 μM), and the enzymatic activities were measured at 3 different time points; 1, 20 and 60 min. The fluorescence emission at 450 nm was obtained using a Perkin Elmer luminescence spectrophotometer LS50B after excitation at 380 nm.

2.2.6 Cathepsin L Activity Assay Kit

Raw264.7 cells were grown in a T175 flask with Dulbecco's modified eagle medium (DMEM) containing 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 200 mM L-glutamine. The cells were detached from the flask using a cell scraper, and collected by centrifugation. Cells (1×10^6) were lysed using the chilled CL cell lysis buffer provided in the cathepsin L Activity Assay Kit (Biovision, Mountain View, CA). The cells were incubated on ice for 10 minutes, centrifuged after at top speed for 5 minutes, and the supernatant was transferred to a black 96-well microplate. 50 μL of cathepsin L reaction buffer to each sample, and 7 different inhibitors were added to the samples. The samples were incubated for 10 minutes with either 10 μM cathepsin L Inhibitor (Ac-FF-FMK, provided by the kit), 10 μM K17, 10 μM E64, 10 μM CA074, 10 μM GM6001, 10 μM 3,4 DCIC, or 500 nM CatSI. The activity assay was initiated by adding 200 μM (final) of the

cathepsin L substrate, Ac-FR-AFC (from the kit) or the analogous substrate, Z-FR-MCA, and incubated at 37°C for 1-2 hours. The samples were read at excitation wavelengths of 485 nm and emission 530 nm using Perkin Elmer luminescence spectrophotometer LS50B. Activities towards both substrates were compared.

2.2.7 Statistics

All measurements were done in triplicates and repeated three times. Data are expressed as the mean \pm standard deviation (SD). When applicable, the Student's unpaired t-test was performed. A probability level of < 0.05 signified a statistically significant difference. All statistics were evaluated using GraphPad Prism, version 5.0 for Mac OS X (GraphPad Software, San Diego, CA).

3 RESULTS

3.1 Elastin and Fibrin Degradation by Cathepsins

The main objective of this aim was to quantify the contribution of individual cathepsins in macrophage mediated elastin degradation, and to determine the capability and potency of cathepsins to hydrolyze fibrin. As substrate for the elastin degradation assays, I used commercially available DQ-elastin which has the advantage of high sensitivity in cell-based assays and due to its solubility allows accurate and easy distribution within the assays. This substrate uses a proprietary fluorescence quenched label which allows detection only after proteolytic degradation of the substrate. I initially evaluated insoluble Congo-Red elastin and fluorescein-elastin which proved insufficiently sensitive in the assays employed (data not shown). It should be noted that extracellular elastin fibers are by nature insoluble and thus soluble elastin preparations are a compromise. They are likely accessible to hydrolysis also by non-elastases as described for DQ-elastin (data sheet of commercially available product by Molecular Probes/Invitrogen, Eugene, OR). The viability of the cells was determined by the LDH cytotoxicity assay to assure no toxicity effects of the inhibitor used in the cell assays. The viability assay showed that the inhibitors were not toxic to the cells, and the concentration was adequate to use in cell-based assays. Fibrin was generated as described in the Methods section.

3.1.1 Quantification of Elastolytic Activities of Cathepsins B, K, L, and S Expressed in Mouse Macrophages

We determined the elastolytic activities of proteases expressed in macrophages using DQ-elastin as substrate. Moreover, we measured the contribution of each cathepsin and other elastases towards elastin degradation by using 8 different protease inhibitors in 4 different types of macrophages including wild-type, cathepsin K^{-/-}, cathepsin L^{-/-}, and Raw264.7 cells.

In figure 3.1.1, cathepsin-related elastolytic activities were compared among 4 different types of macrophages using 3 inhibitors including 10 μM K17, 500 nM CatSI, and 10 μM CA074. K17 is an irreversible vinyl sulfone inhibitor of cysteine proteases, which allowed the estimation of total elastolytic activities of all cathepsins expressed in macrophages. 10 μM K17 reduced the DQ-elastin degradation by approximated 40% in wild-type, cathepsin K^{-/-}, and Raw264.7 macrophages, thus representing the total contribution of all cathepsins towards elastin degradation. Interestingly, cathepsin L^{-/-} macrophages showed ~80% reduction in the elastolytic activity with 10 μM K17, which was observed to be twice the reduction of the other macrophages. This implied that cathepsin L^{-/-} macrophages express twice the amount of cathepsins that are enrolled in elastin degradation; further details will be discussed in the discussion part.

CatSI is a potent and selective inhibitor for cathepsin S, which permitted the estimation of cathepsin S towards elastin degradation. At 500 nM CatSI, no inhibition was observed in

wild-type and Raw264.7 macrophages. In cathepsin K^{-/-} and cathepsin L^{-/-} macrophages, a 10% and 40% reduction was observed, respectively. The results indicate that cathepsin S does not play a major role in elastin degradation in wild-type and Raw264.7 macrophages. However, in cathepsin K and L-deficient macrophages, cathepsin S played a significant role in elastin degradation. Therefore, these results suggest the possibility of up-regulation of cathepsin S in these macrophages in order to compensate for the loss of these cathepsins.

CA074 is a selective irreversible inhibitor of extracellular cathepsin B. CA074 at a concentration of 10 µM inhibited the DQ-elastin degrading macrophage activity by ~10% in wild-type and cathepsin K^{-/-} macrophages, which implied that cathepsin B contributes approximately 10% towards elastolysis. There was a ~40% reduction in the elastin degradation in cathepsin L^{-/-} macrophages, and no significant change was observed in Raw264.7 macrophages. This implies that in cathepsin L^{-/-} macrophages, there was a dramatic increase in the contribution of cathepsin B towards elastin degradation, which may be again due to an increased compensatory expression of cathepsin B in cathepsin L^{-/-} macrophages compared to the wild-type macrophages.

In summary, mouse wild-type macrophages showed 40% of cathepsin-mediated elastin degradation as shown by the treatment with K17. Out of this 40% cathepsin-mediated elastin degradation, cathepsin S did not play a role in elastin degradation since no reduction was observed with CatSI treatment. Cathepsin B contributed 10% of the elastolytic activity. Therefore, the remaining 30% can be said to be the elastolytic activities of cathepsin K and cathepsin L.

In cathepsin-deficient macrophages, a compensatory increase in the activities of related cysteine proteases was observed. Cathepsin K^{-/-} macrophages revealed a ~40% reduction of the elastolytic activity in the presence of 10 μM K17, which represents the elastolytic activities of all cathepsins including cathepsin B, L, and S. Each, cathepsin B and cathepsin S contributed about 10% towards the total cathepsin-dependent elastin degradation. The remaining 20% is assumed to be the contribution of cathepsin L in macrophages. In cathepsin L^{-/-} macrophages, 80% of elastolytic activity was due to the contribution of cathepsins as shown by the treatment with K17. Each, cathepsin B and cathepsin S contributed 40% towards the degradation of soluble elastin based on the effects of the cathepsins B and S inhibitors. This suggests a major shift in the profile of cathepsin expression in these cells towards cathepsins B and S. On the other hand, cathepsin K had a negligible contribution to elastin turnover in cathepsin L-deficient macrophages.

In Raw264.7 macrophages, 10 μM K17 reduced the elastin degradation by approximately 40%, which represented the contribution of cathepsins B, K, L, and S. CatSI and CA074 had no effect on elastin degradation meaning that cathepsin S and cathepsin B do not play a significant role; rather the 40% reduction can be said to be due to cathepsin K and L.

Contribution of individual cathepsins towards elastolysis is summarized in Table 3.1.1

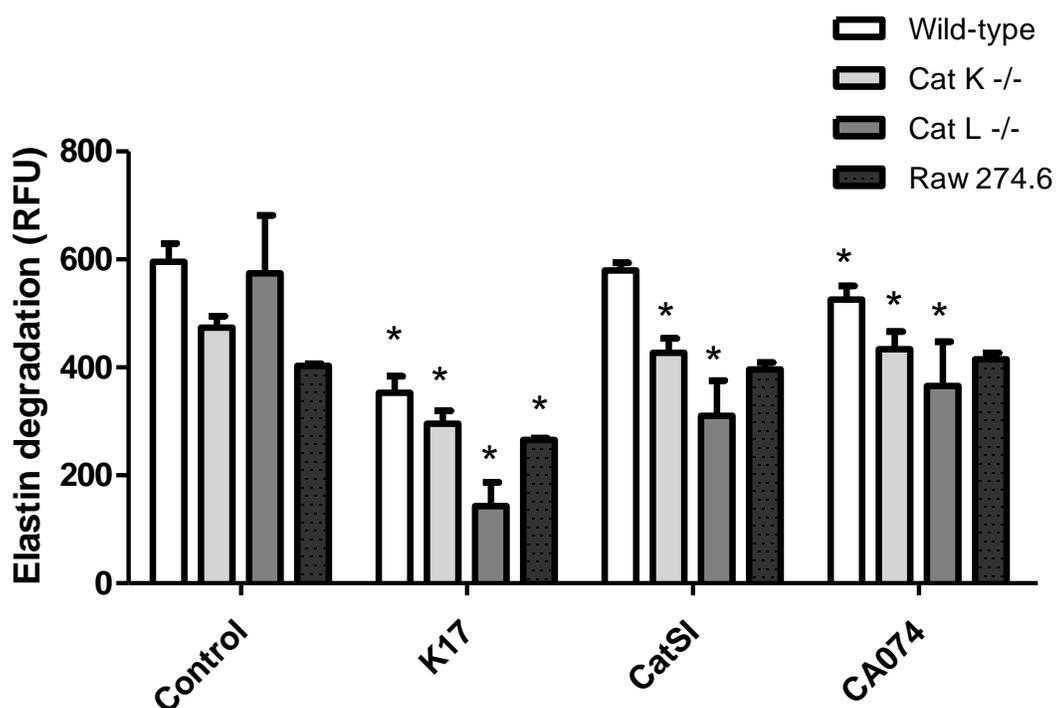


Figure 3.1.1 Degradation of DQ-elastin by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages. DQ-elastin degradation was inhibited by protease inhibitors. All cathepsins were inhibited by 10 μ M K17. Cathepsin S was inhibited with 500nM CatSI, and cathepsin B was inhibited at 10 μ M CA074. Error bars represent mean \pm SD from three independent experiments.

	wild-type	cathepsin K ^{-/-}	cathepsin L ^{-/-}	Raw264.7
Control	100 %	100 %	100 %	100 %
K17	40% reduction	40% reduction	80% reduction	40% reduction
CatSI	0% reduction	10% reduction	40% reduction	0% reduction
CA074	10% reduction	10% reduction	40% reduction	0% reduction

Table 3.1.1 Summary of DQ-elastin Degradation by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages and the Inhibitory Effects of K17, CatSI, and CA074.

3.1.2 Quantification of Elastolytic Activities of Extra- and Intracellular Cathepsins Expressed in Mouse Macrophages

Cathepsins are lysosomal cysteine proteases, which are also secreted into the extracellular environment. The aim of this research was to quantify the extra and intracellular contributions of cathepsins to the overall elastin degradation.

Figure 3.1.2 and Table 3.1.2 show the contributions of extra- and intracellular cathepsin activities to elastin degradation by employing extra- or intracellularly active inhibitors, which include E64, E64d, CA074, and CA074-Ome. E64 (non-cell permeable) and E64d (cell-permeable) are the epoxysuccinyl-based irreversible inhibitors, which are used for general inhibition of cysteine cathepsins. The effects of E64 should represent the inhibition of extracellular cathepsin activities, whereas E64d should represent the suppression of intracellular cathepsin activities. E64d and CA074-Ome are pro-drugs which require the cleavage of the ester bond by intracellular esterases for activation. E64d and CA074-Ome are thus thought to act exclusively intracellularly. Furthermore, intracellular fluorescence was measured using DQ-elastin, which demonstrated the phago/pinocytosis of elastin by macrophages (Figure 3.1.3).

In wild-type and cathepsin K^{-/-} macrophages, 10 μM E64 mediated inhibition reduced total DQ-elastin degradation by ~20%, whereas 10 μM E64d inhibited approximately 30% of the elastolytic activity. These data implied that ~20% of the elastin degradation was due to extracellular cathepsins, and ~30% were due to intracellular cathepsins. Cathepsin L^{-/-}

macrophages showed 60% reduction with 10 μ M E64 and 10 μ M E64d, respectively. In other words, there was 60% of cathepsin-mediated intracellular elastin degradation, and another 60% of cathepsin-mediated extracellular elastin degradation which suggests that there must be some cross-inhibitory activities for both types of compounds.

E64 reduced the DQ-elastin degradation by only ~20% in wild-type macrophages, whereas a ~60% reduction was observed in cathepsin L^{-/-} macrophages. This suggested that there might be an up-regulation of other cathepsins in cathepsin L-deficient cells that compensates for the loss of cathepsin L. However, this will require further investigation. Lastly, Raw264.7 macrophages showed a ~30% decrease in elastin degradation in the presence of E64, and another ~20% reduction of elastinolysis in the E64d-treated macrophages.

CA074 (non-membrane permeable) and CA074-Ome (membrane permeable prodrug) are irreversible and selective inhibitors of cathepsin B. CA074 is able to inactivate extracellular cathepsin B activity as it is not able to traverse the cell membrane due to the negatively charged group. On the other hand, the methylated version, CA074-Ome, can penetrate the cell membrane and act upon intracellular cathepsin B activity.

In wild-type macrophages, 10 μ M CA074 and 10 μ M CA074-Ome were able to reduce the elastolytic activity by approximately 10% each, which implied that extra- and intracellular cathepsin B have contributed ~20% towards elastin degradation. In cathepsin K^{-/-} macrophages, we demonstrated that 10 μ M CA074 reduced elastolytic activity by ~10%,

whereas 10 μ M CA074-Ome had no significant reduction in elastin degradation. In contrast, there was approximately 30-40% reduction with each CA074 and CA074-Ome in cathepsin L^{-/-} macrophages. When compared to wild-type or cathepsin K^{-/-} macrophages, the reduction percentage of elastin degradation increased about 20-30%. This result is also in parallel with the assumption made previously with E64 and E64d. Cathepsin L deficiency may have caused an up-regulation of extra- and intracellular cathepsin B activities. With Raw264.7 macrophages, there was no significant reduction in the CA074 and CA074-Ome mediated elastin degradation. This result suggested that there are negligible amount of cathepsin B expressed in Raw264.7 macrophages that plays a role in elastin degradation.

For comparison, K17 which acts intra as well as extracellularly and which is more potent than the epoxysuccinate peptide derivatives showed a 40 and 80% inhibition for the wild-type and cathepsin-deficient macrophages, respectively. However, the combined inhibition effects of the intra- and extracellular acting E64 inhibitors surpassed the effect of K17 and even would end up with a more than 100% inhibition of the elastolytic activities in cathepsin L-deficient macrophages. This is unlikely and thus I speculate that also E64 has intracellular activity. Therefore, I examined the intracellular fluorescence upon uptake of DQ-elastin by macrophages and in the presence of either an extra- or intracellular inhibitor. To visualize intracellular degradation of DQ-elastin, Raw264.7 macrophages were treated with inhibitors (E64, E64d, CA074, and CA074-Ome) and incubated with DQ-elastin as described above. DQ-elastin generates green fluorescence upon proteolytic cleavage. In figure 3.1.3, intracellular fluorescence was observed in Raw264.7 macrophages confirming the uptake of DQ-elastin for intracellular degradation. The fluorescent intensities of E64-

treated and E64d-treated macrophages showed no significant differences. Similarly, intracellular fluorescence of CA074-treated and CA074-Ome treated Raw264.7 macrophages were not significantly different from each other. This indicates that both types of inhibitors (membrane and non-membrane permeable) have similar effects on intracellular cathepsin activities. It is likely that the “extracellular type” inhibitors are taken up by phago/pinocytosis together with the DQ-elastin and inhibit intracellular cathepsin activity.

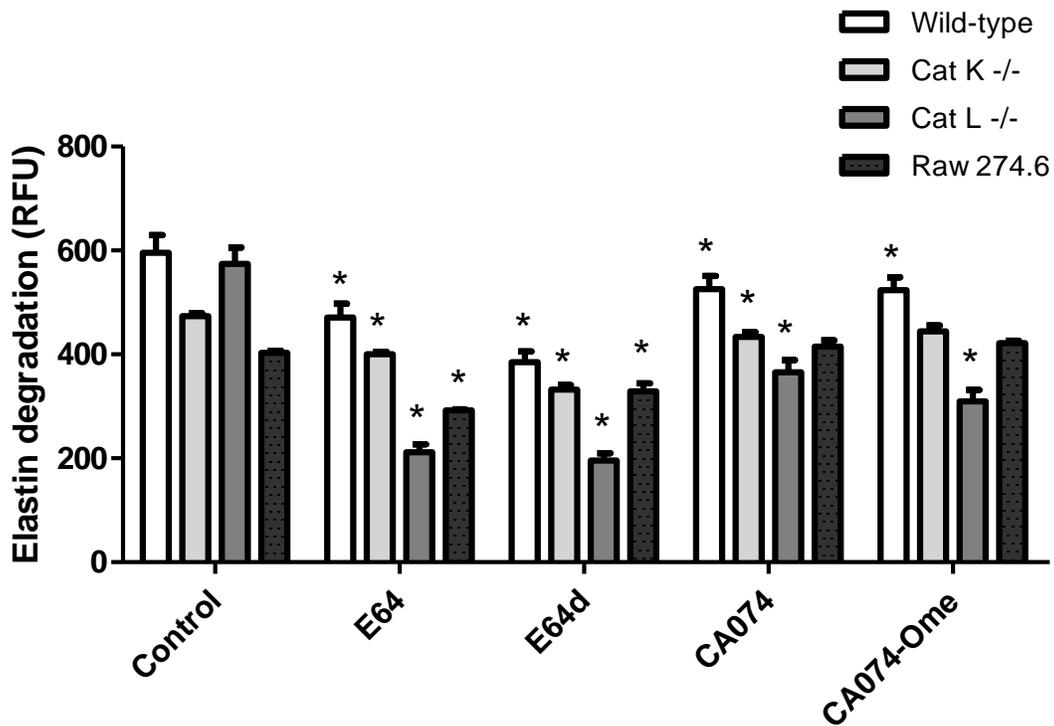


Figure 3.1.2 Degradation of DQ-elastin by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages. DQ-elastin degradation was inhibited by protease inhibitors.

Extracellular and intracellular cathepsin activity was inhibited by 10 μM E64 and 10 μM E64d, respectively. Extracellular and intracellular cathepsin B activity was inhibited by 10 μM CA074 and 10 μM CA074-Ome, respectively. Error bars represent mean ± SD from three independent experiments.

	wild-type	cathepsin K ^{-/-}	cathepsin L ^{-/-}	Raw264.7
Control	100 %	100 %	100 %	100 %
E64	20% reduction	20% reduction	60% reduction	30% reduction
E64d	30% reduction	30% reduction	60% reduction	20% reduction
CA074	10% reduction	10% reduction	30% reduction	0% reduction
CA074-Ome	10% reduction	0% reduction	40% reduction	0% reduction

Table 3.1.2 Summary of DQ-elastin Degradation by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages and the Inhibitory Effects of E64, E64d, CA074, and CA074-Ome.

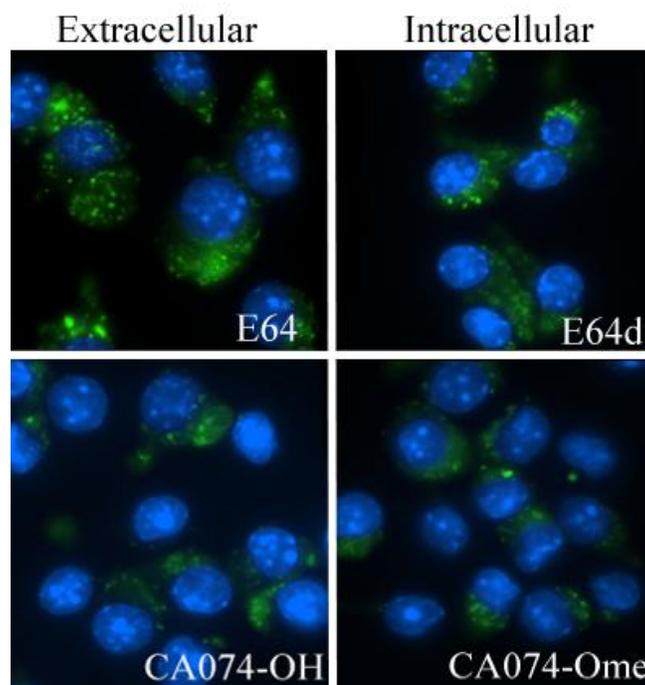


Figure 3.1.3. Intracellular Degradation of DQ-elastin by Raw264.7 Macrophages and the Effect of Intra- and Extracellular Cathepsin Inhibitors. Top panel: 10 μ M E64 and 10 μ M E64d. Bottom panel: 10 μ M CA074 and 10 μ M CA074-Ome.

3.1.3 Quantification of Elastolytic Activities of Non-Cysteine Proteases Expressed in Mouse Macrophages

In figure 3.1.4 and in table 3.1.3, I have explored the contribution of non-cysteine proteases towards elastin degradation by utilizing non-cysteine inhibitors such as GM6001 and 3,4-DCIC. GM6001 is a reversible non-specific hydroxamic acid-based matrix metalloproteinase (MMP) inhibitor. 10 μ M GM6001 allowed the estimation of MMP contribution towards elastin degradation in 4 different types of macrophages. 3,4-DCIC, also known as 3,4-dichloroisocoumarin (3,4-DCIC), is a general inhibitor for serine proteases. The serine protease inhibitor allowed the quantification of the contribution of serine proteases towards total elastin turnover.

In wild-type and cathepsin K^{-/-} macrophages, there was a 10% decrease in DQ-elastin degradation with the treatment of GM6001, which implied that MMPs contribute ~10% towards elastin fragmentation. There was no significant reduction in cathepsin L^{-/-} and Raw264.7 macrophages, which implies negligible roles of MMPs in elastin degradation. In summary, these results suggest that MMPs have only a minor role in elastin degradation in macrophages and do not seem to be up-regulated in cathepsin deficiencies.

There was a 20% reduction in elastin degradation with 10 μ M 3,4-DCIC in wild-type, cathepsin K^{-/-}, and Raw264.7 macrophages suggesting that serine proteases contribute 20% to the overall elastolytic activities. In cathepsin L^{-/-} macrophages, no reduction in elastolysis was observed with the treatment of 3,4-DCIC indicating that serine proteases

neither play a significantly role or are up-regulated in these cells.

I have also demonstrated the effect of treating the macrophages with inhibitor cocktails. K17, GM6001, and 3,4-DCIC were applied all-together in order to suppress the total elastolytic activities. However, the elastin degradation was not completely suppressed suggesting there are other factors that give rise to elastin degradation other than cysteine, serine, and metalloproteinases.

With the combined treatment using K17+GM6001+3,4-DCIC together the elastolytic activity was reduced by ~60-70% in wild-type and cathepsin K^{-/-} macrophages; this result represents the total contributions of cathepsins, MMPs, and serine proteases. In figure 3.1.1, K17 alone reduced the elastolytic activities by ~40%, MMP alone reduced ~10%, and 3,4-DCIC alone reduced 20% shown in figure 3.1.4, which adds up to be ~70% and thus in the same range as the combination treatment.

In cathepsin K^{-/-} macrophages, ~50% of the elastolytic activity was decreased with the treatment of K17, GM6001, and 3,4-DCIC at once. This strongly supports the assumption that cathepsins, MMPs, and serine proteases contribute ~50 % towards elastin degradation. In contrast, there was a 40% decrease with the treatment of K17 only (figure 3.1.1), a 10% decrease with GM6001 only (figure 3.1.4), and another 20% reduction with 3,4-DCIC only (figure 3.1.4), which all together adds up to ~70%. There is approximately 20% difference between all-in-one treatment, and individual inhibitor treatment. The reason for this discrepancy is not clear but it can be speculated that the inhibitor cocktail may lead to a

stronger up-regulation of inhibitor insensitive proteases than the inhibition by individual drugs.

In cathepsin L^{-/-} macrophages, there was a dramatic depression in the DQ-elastin degradation with the combined inhibitor treatment when compared to the wild-type or cathepsin K deficient macrophages. 80% reduction assumed to be due to the up-regulation of elastases in cathepsin L^{-/-} macrophages. Interestingly, 80% reduction was observed with K17-inhibited elastin degradation (figure 3.1.1). GM6001 and 3,4-DCIC had no additional effect on elastin degradation. Applying K17, GM6001, and 3,4-DCIC at once and applying inhibitors separately had no differences. They both had ~80% inhibition towards elastases expressed in cathepsin L-deficient macrophages.

In Raw264.7 macrophages, there was a 40% reduction with the combined inhibitor treatment revealing the total involvement of cathepsins, MMPs, and serine proteases in elastin degradation. K17 alone reduced ~40% of its elastolytic activities, and 3,4-DCIC reduced another 20% (figure 3.1.4), which adds up to be ~60%. A speculation for this discrepancy was suggested above, where the inhibitor cocktail may have led to stronger stimulation in up-regulating other proteases. However, additional investigations are required to confirm the up-regulation of other proteases.

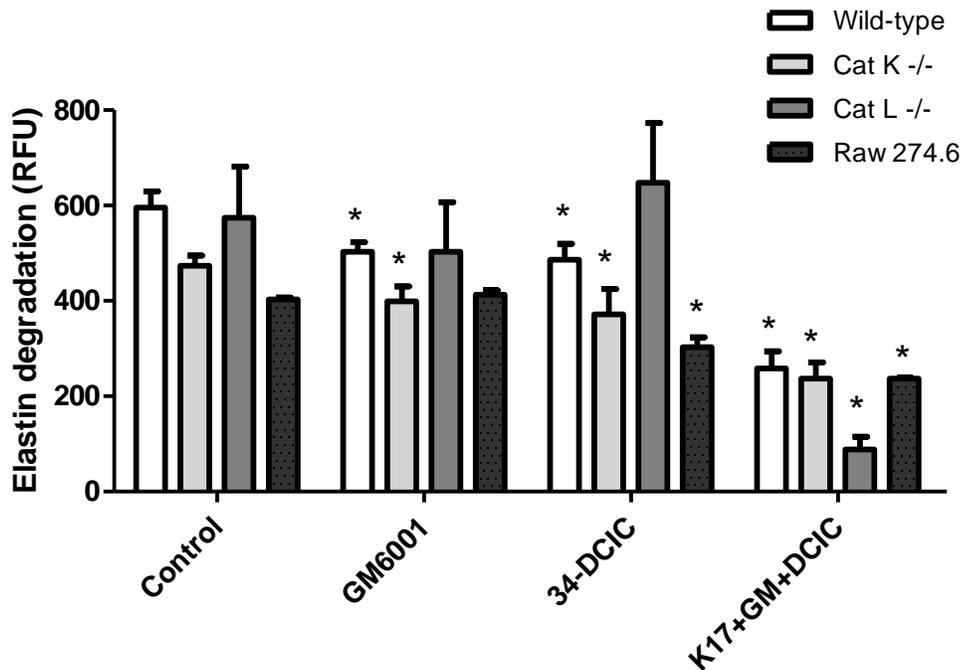


Figure 3.1.4 Degradation of DQ-elastin by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages. DQ-elastin degradation was inhibited by protease inhibitors. All MMPs were inhibited at 10 μM GM6001, and all serine proteases were inhibited at 10 μM 3, 4-DCIC. All proteases were inhibited at 10 μM K17, 10 μM GM6001, and 10 μM 3, 4-DCIC combined. Error bars represent mean ± SD from three independent experiments.

	wild-type	cathepsin K ^{-/-}	cathepsin L ^{-/-}	Raw264.7
Control	100 %	100 %	100 %	100 %
GM6001	10% reduction	10% reduction	0% reduction	0% reduction
3,4-DCIC	20% reduction	20% reduction	0% reduction	20% reduction
K17 + GM6001 + 3,4 -DCIC	60% reduction	50% reduction	80% reduction	40% reduction

Table 3.1.3 Summary of DQ-elastin Degradation by Wild-type, Cathepsin K^{-/-}, Cathepsin L^{-/-}, Raw264.7 Macrophages and the Inhibitory Effects of GM6001, 3,4-DCIC, and K17+GM6001+3,4-DCIC Cocktail.

3.1.4 Characterization of Cathepsins B, K, L, and V in Fibrinolysis

In the first part of my studies, the role of cathepsins expressed in macrophages towards elastin degradation has been characterized. Here, I investigated the involvement of cathepsins in fibrinolysis and their efficacy compared to the fibrin degrading enzyme, plasmin. 3 μ M fibrinogen and 3 nM thrombin were used to generate fibrin clots and they were incubated with 400 nM of each plasmin, cathepsins B, K, L, and V. The half-lysis time of fibrin clots were measured and compared among plasmin and cathepsins.

In figure 3.1.5, the half-lysis time for plasmin was approximately 70 minutes, which was found to be the shortest among 5 enzymes used in this assay. The half-lysis time for cathepsin B (300 minutes) was 4-times longer than plasmin which implied that cathepsin B is 4-times less efficient in fibrinolysis. Moreover, cathepsin B was shown to be the most inefficient enzyme in fibrin degradation among cathepsins K, L, and V. Cathepsin K and L revealed the half-lysis time of 110~120 minutes which was slightly longer than plasmin and shorter than cathepsin B. The half-lysis time of cathepsin V was approximately 200 minutes which was shown to be shorter than cathepsin B but longer than plasmin, cathepsin K and L. The fibrinolytic activity of cathepsin V was 2~3-fold lower when compared with plasmin. The order of preference in fibrin degradation follows: plasmin > cathepsin L > cathepsin K > cathepsin V > cathepsin B.

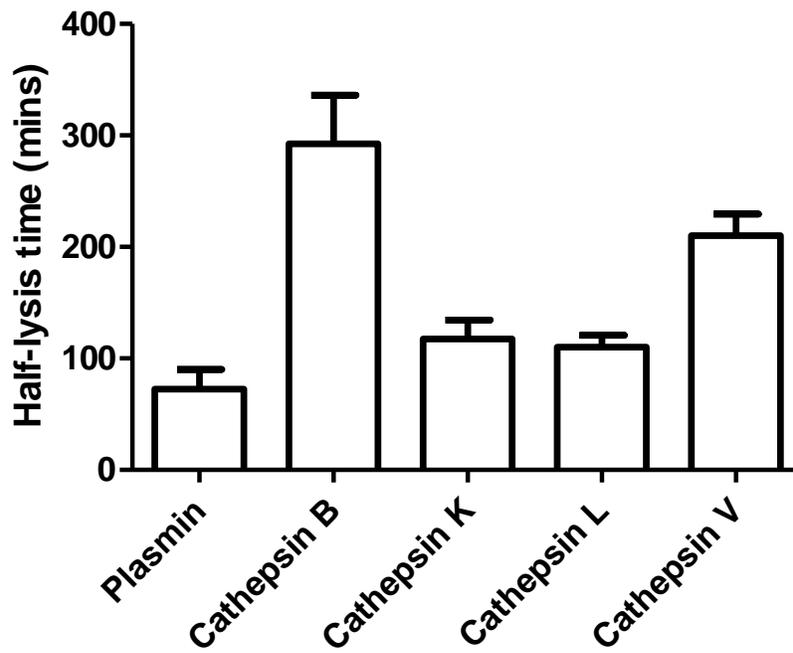


Figure 3.1.5 Half-lysis Time of Fibrin Clots by 400 nM Cathepsins B, K, L, V, and Plasmin. Error bars represent mean \pm SD from two independent experiments.

3.2 Measurement of Cathepsin Activity using Peptidyl Substrates

Peptidyl substrates with either a chromogenic or fluorogenic leaving group are widely used in protease biochemistry. They allow the determination of protease activities in biological samples or are used to determine the kinetic parameters of inhibitors and substrate specificity determinants. *In vivo*, most proteases work below their maximal velocity (V_{max}) which is in or below the appropriate K_m range of the substrates. At very high substrate concentrations (about $>5-10 \times K_m$), most proteases reach their maximal velocity. As most cathepsins have K_m values for their peptide substrates between 5-100 μM , I choose two substrate concentration (5 μM : $S < K_m$ and 200 μM : $S \gg K_m$) to determine the specificity of cathepsins B, K, L, S, and V for their respective substrates. The K_m and k_{cat}/K_m values for the substrates tested are displayed in Table 3.2.1. Based on previously determined k_{cat}/K_m values for individual substrates, commercial substrate assays have been developed to identify single cathepsin activities in biological samples. The original aim of my studies was to use these assay kits to quantify cathepsin activities in macrophages cell extracts. However, my results mostly invalidated those assays as they are significantly less specific as claimed by the vendors.

Enzyme	Substrate	kcat (s⁻¹)	Km (μM)	kcat/Km (M⁻¹ s⁻¹)
Cathepsin B	Z-RR-MCA	20.0 ± 6.0	470 ± 90	43000 ± 7600
Cathepsin K	Z-RR-MCA	0.0025 ± 0.0005	25.0 ± 0.8	100
Cathepsin L	Z-RR-MCA	0.07 ± 0.008	40.0 ± 10.0	1750
Cathepsin S	Z-RR-MCA	0.020 ± 0.001	220. ± 60	91
Cathepsin V	Z-RR-MCA	0.011 ± 0.001	168 ± 26	65
Cathepsin B	Z-LR-MCA			
Cathepsin K	Z-LR-MCA	1.42 ± 0.01	4.2 ± 0.9	341756
Cathepsin L	Z-LR-MCA	1.05 ± 0.2	12.7 ± 2.7	90551
Cathepsin S	Z-LR-MCA	5.6 ± 1.8	23 ± 5	243000
Cathepsin V	Z-LR-MCA	0.41 ± 0.05	4.5 ± 1.4	91111
Cathepsin B	Z-FR-MCA	77.8 ± 16.0	188 ± 41	424000 ± 91000
Cathepsin K	Z-FR-MCA	0.73 ± 0.2	7.4 ± 0.8	98648
Cathepsin L	Z-FR-MCA	1.16 ± 0.3	0.8 ± 0.1	1446118
Cathepsin S	Z-FR-MCA	1.9 ± 0.2	22 ± 2	86400
Cathepsin V	Z-FR-MCA	0.71 ± 0.06	6.4 ± 1.5	110937
Cathepsin B	Z-VVR-MCA			
Cathepsin K	Z-VVR-MCA			
Cathepsin L	Z-VVR-MCA			
Cathepsin S	Z-VVR-MCA	15 ± 2	18 ± 2	830000
Cathepsin V	Z-VVR-MCA	0.15 ± 0.01	8.1 ± 0.8	18520

Table 3.2.1 Kinetic Parameters for the Hydrolysis of Various MCA Substrates by Cathepsin B, Cathepsin K, Cathepsin L, Cathepsin S, and Cathepsin V. Values were taken from *Fox et al 1995, Lecaille et al 2002, Bromme et al 1994, Bromme et al 1999.*

[70-74]

3.2.1. Selectivity of Cathepsins towards Different Substrates At or Below K_m

Concentrations (5 μM)

The substrate specificities of five different cathepsins were determined at 5 μM concentrations of four different fluorescence-labeled peptide substrates (Z-RR-MCA, Z-LR-MCA, Z-FR-MCA, and Z-VVR-MCA) which have been described to be specific for the following cysteine proteases: cathepsin B, cathepsin K, cathepsin L, and cathepsin S. Here, I show that Z-RR-MCA and Z-VVR-MCA are relatively specific for cathepsin B and cathepsin S, respectively. However, Z-LR-MCA and Z-FR-MCA are non-specific substrates, and are hydrolyzed by all five cathepsins with different efficiency.

As shown in figure 3.2.1a, cathepsin B had maximal catalytic activity toward the dipeptide substrate Z-RR-MCA at 5 μM substrate concentration, which has been known as a cathepsin B specific substrate. The hydrolysis rate of Z-RR-MCA by cathepsin L was significantly lower compared to cathepsin B. Other cathepsins showed effectively no activity towards this substrate. It can be concluded that Z-RR-MCA is a relatively selective substrate for cathepsin B at non saturating substrate concentration.

In figure 3.2.1b, Z-LR-MCA, a substrate suggested as specific for cathepsin K was efficiently hydrolyzed by cathepsin K. However, cathepsin V showed 30% higher substrate hydrolysis rate compared to cathepsin K. Other enzymes also revealed acceptable proteolytic activity towards Z-LR-MCA. For example, cathepsin L showed about 50% of the cathepsin V activity and cathepsin S was about 20% of the cathepsin V activity with

this substrate. The order of preference for Z-LR-MCA hydrolysis was cathepsin V > cathepsin K > cathepsin L > cathepsin S > cathepsin B. Consequently, the cathepsin K specific substrate, Z-LR-MCA, was hydrolyzed by cathepsin V with the most potent enzymatic activity and cathepsin K activity towards Z-LR-MCA was 30% less efficient than cathepsin V. Here, it can be concluded that Z-LR-MCA is a non-specific substrate that is hydrolyzed by cathepsins B, K, L, S, and V with overall comparable efficiencies.

The synthetic substrate, Z-FR-MCA, is widely used to measure cathepsin L activity as it possesses the cathepsin L preferred P2 phenylalanine residue. Cathepsin L hydrolyzed the substrate, Z-FR-MCA, with the best efficiency (figure 3.2.1c). Cathepsin V was also capable of catalyzing the peptidyl substrate with a similar efficiency as cathepsin L. Other cathepsins such as cathepsin B, cathepsin K, and cathepsin S were able to hydrolyze the substrate with ~80% less efficient manner compared to cathepsins L and V. Therefore, the substrate, Z-FR-MCA, cannot be a cathepsin L specific substrate, rather it is a relatively competent substrate for all cathepsins.

In figure 3.2.1d, the tripeptide fluorogenic substrate, Z-VVR-MCA, also known as the most sensitive substrate available for cathepsin S, was hydrolyzed by cathepsin S with highest efficiency. Cathepsins B, V and L revealed relatively low activity, which were only ~10% active towards Z-VVR-MCA compared to the activity of cathepsin S. Cathepsin K revealed negligible or no detectable activity with the tripeptide substrate. Here it can be concluded that Z-VVR-MCA is a relatively specific substrate for cathepsin S.

In summary, at below V_{max} conditions ($S = 5\mu M$) as normally seen under *in vivo* conditions, only the cathepsin B and with some limitation the cathepsin S substrate displayed a sufficient degree of specificity. In contrast, the so-called cathepsin L and K substrates must be considered as non-specific.

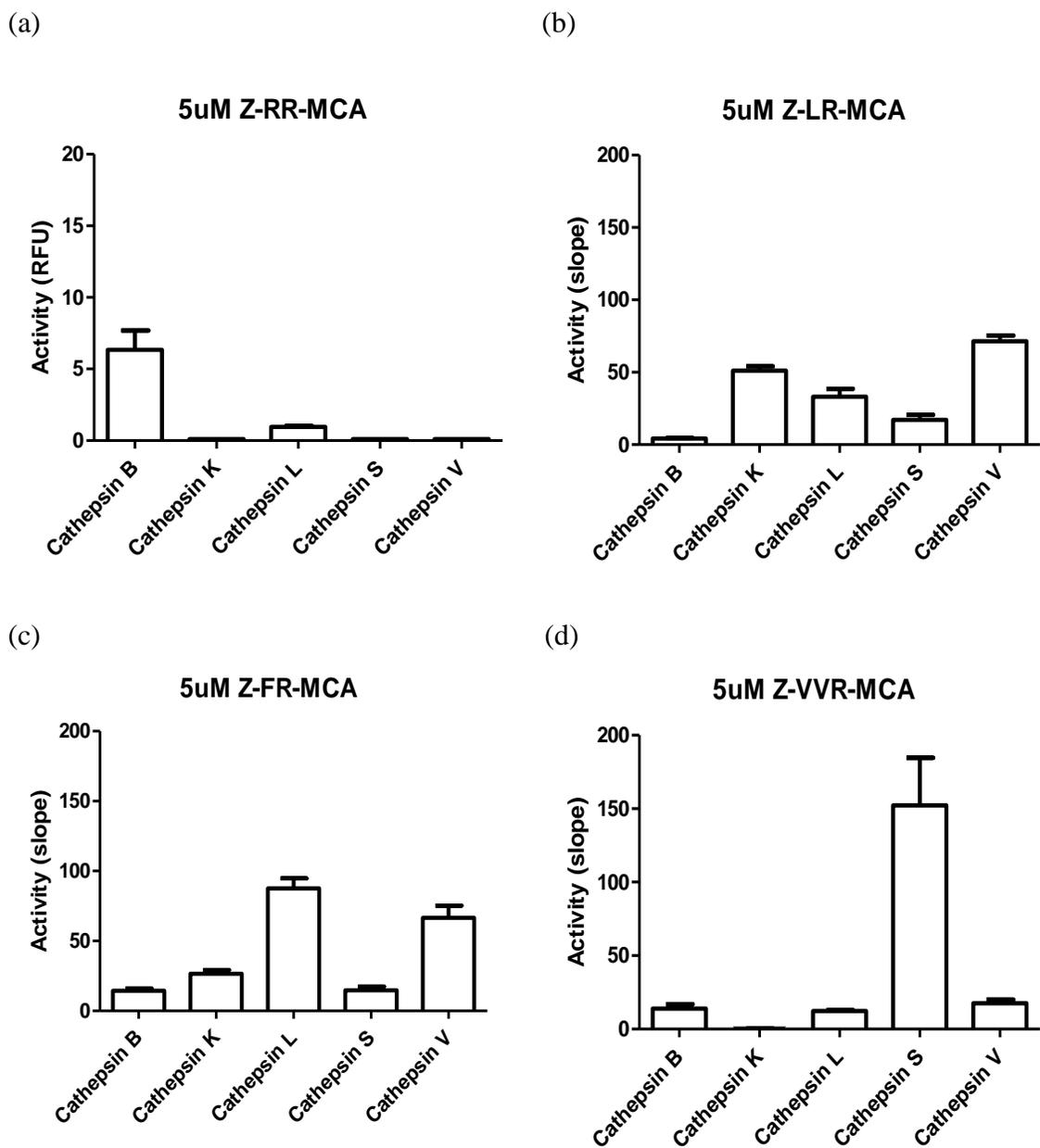


Figure 3.2.1 Enzymatic Activities of Purified Cathepsins B, K, L, S, and V were Measured Using 4 Different Peptidyl Substrates. (a) 5 μM Z-RR-MCA, (b) 5 μM Z-LR-MCA, (c) 5 μM Z-FR-MCA, and (d) 5 μM Z-VVR-MCA. The pH of activity buffer was kept at 5.5 in order to measure the optimal activities of recombinant cathepsins. Error bars represent mean ± SD from three independent experiments.

3.2.2 Selectivity of Cathepsins towards Different Substrates at V_{max} Concentrations (200 μM)

The proteolytic activities of cathepsins B, K, L, S, and V were monitored by the hydrolysis of 4 fluorogenic substrates including Z-RR-MCA, Z-LR-MCA, Z-FR-MCA, and Z-VVR-MCA at 200 μM concentration. 200 μM is the substrate concentration at which most of the cathepsins reach their maximal velocity of hydrolysis and as it is recommended by the manufacturer's assay protocol. Moreover, as the recommended assay time is 60 min, the high substrate concentration is thought to prevent a premature depletion of the substrate. Here, I show the effect of high substrate concentration towards cathepsin activity and how the excess substrate concentration can eventually lead to the inhibition of enzymatic activities of certain cathepsins.

In figure 3.2.2a, the Z-RR-MCA at a concentration of 200 μM was hydrolyzed by 5 different cathepsins including cathepsins B, K, L, S, and V. Cathepsin B showed the highest catalytic activity towards Z-RR-MCA at 200 μM substrate concentrations, whereas other cathepsins showed negligible catalytic activity towards the substrate. When we compared the enzyme activity at 5 μM and 200 μM substrate concentration, the activity of cathepsin B was at least 10-fold higher. Cathepsin L and cathepsin V displayed negligible but significant increase in the catalytic activity (4~10-fold) with increased substrate concentration. Cathepsins K and S revealed no enzymatic activities at both, 5 μM and 200 μM , substrate concentration.

As expected, the hydrolysis of Z-LR-MCA was increased in cathepsins B, S, and V with the increased substrate concentration (figure 3.2.2b). Cathepsin B showed a 22-fold increase, cathepsin S a 16-fold increase, and cathepsin V revealed a 1.5-fold increase in parallel with a 40-fold increase in the substrate concentration. In contrast, cathepsins K and L unveiled a significant reduction in their hydrolysis rates with elevated substrate concentration. Z-LR-MCA is known as a cathepsin K sensitive substrate, but the dramatic increase in substrate concentration from 5 μM to 200 μM led to a significant reduction in cathepsin K and L activities. Consequently, at the recommended 200 μM substrate concentration, cathepsin K was no longer the best hydrolase for Z-LR-MCA, rather cathepsin S was more efficient. At 5 μM substrate concentration, cathepsin V was the best enzyme for Z-LR-MCA. This showed that Z-LR-MCA is not an adequate substrate to measure the activity of cathepsin K at conditions recommended by the providers of the assay kits. At 200 μM Z-LR-MCA, the fluorogenic substrate was even less specific than at 5 μM substrate concentration.

In figure 3.2.2c, the hydrolysis of the cathepsin L preferred substrate, Z-FR-MCA, by cathepsins B, K, L, S, and V were compared at two different concentrations, 5 μM and 200 μM . Cathepsin B and cathepsin S showed 6-fold and 10-fold increased proteolytic activities in parallel to increased substrate concentration. Cathepsin V revealed no significant change in the enzymatic activities with 40-fold elevated substrate concentration. Similar to Z-LR-MCA, the hydrolysis rates by cathepsins K and L dropped significantly when compared to the 5 μM concentration. Cathepsins K and L showed complete loss of peptidolytic activities against Z-FR-MCA at 200 μM indicating a substrate inhibition effect.

Figure 3.2.2d shows the hydrolysis of Z-VVR-MCA at 5 μM and 200 μM for the five cathepsins. Cathepsin S showed the highest proteolytic efficiency compared to other four cathepsins. The enzymatic activity of cathepsin S increased by 10-fold when the concentration of Z-VVR-MCA was altered from 5 μM to 200 μM . A relatively slight increase of enzymatic activities was observed with cathepsins B and V. In contrast, cathepsin L showed no change in the enzymatic activity with increased substrate concentration, and cathepsin K possessed no measurable activity towards the substrate at 5 μM and 200 μM .

The inhibitory effects at high substrate concentrations led me to explore the substrate concentration-dependent inhibition of cathepsins in more detail. I have selected 3 cathepsins including cathepsin K, L, and V and tested with 3 peptidyl substrates including Z-LR-MCA, Z-FR-MCA, and Z-VVR-MCA at various substrate concentrations to quantify substrate inhibitions (figure 3.2.3).

Figure 3.2.3a shows the hydrolase activity of cathepsin K with increasing concentrations of 3 substrates (Michaelis-Menten kinetics). The results showed that the rate of hydrolysis of Z-LR-MCA ($K_m=4.2 \mu\text{M}$) and Z-FR-MCA ($K_m=7.4 \mu\text{M}$) was substantially reduced at high substrate concentrations in the range of 50 μM ~ 200 μM , which did not follow the simple Michaelis-Menten kinetics. The maximal catalytic activity was observed at substrate concentration of 50 μM , which is approximately 10-times the K_m value for Z-LR-MCA and Z-FR-MCA. At 100 μM Z-FR-MCA, the catalytic activity was decreased by ~50%, and at 200 μM the activity was completely lost. Cathepsin K did not show any activity

towards Z-VVR-MCA at increasing substrate concentration ranging from 5 μM to 200 μM .

In figure 3.2.3b, the hydrolysis rate of cathepsin L towards 3 substrates is shown. The rate of hydrolysis was constantly decreased as the substrate concentration increased from 5 μM to 200 μM . The catalytic activity of cathepsin L decreased by 80~90% when the substrate concentration was increased from 5 μM to 50 μM .

The results shown in figure 3.2.3c revealed simple Michaelis-Menten kinetics for cathepsin V with the exception of Z-FR-MCA ($K_m=6.4 \mu\text{M}$). For Z-LR-MCA ($K_m=4.5\mu\text{M}$) and Z-VVR-MCA, the rate of hydrolysis constantly increased until it reached V_{max} , and it plateau after 50 μM substrate concentration. The reaction rate of cathepsin V increased constantly from 5 μM to 100 μM Z-FR-MCA. Then, the activity of cathepsin V was reduced by 50% at 200 μM Z-FR-MCA showing the possible effect of substrate inhibition.

In summary, it was assumed that the increase in substrate concentration will lead to increased hydrolase activity of cathepsins as they would work under V_{max} conditions based on their K_m values. However, this was only observed with cathepsins B and S. Cathepsins K and L showed substantial substrate/product inhibition which would further invalidate the suitability of these assays as enzyme specific.

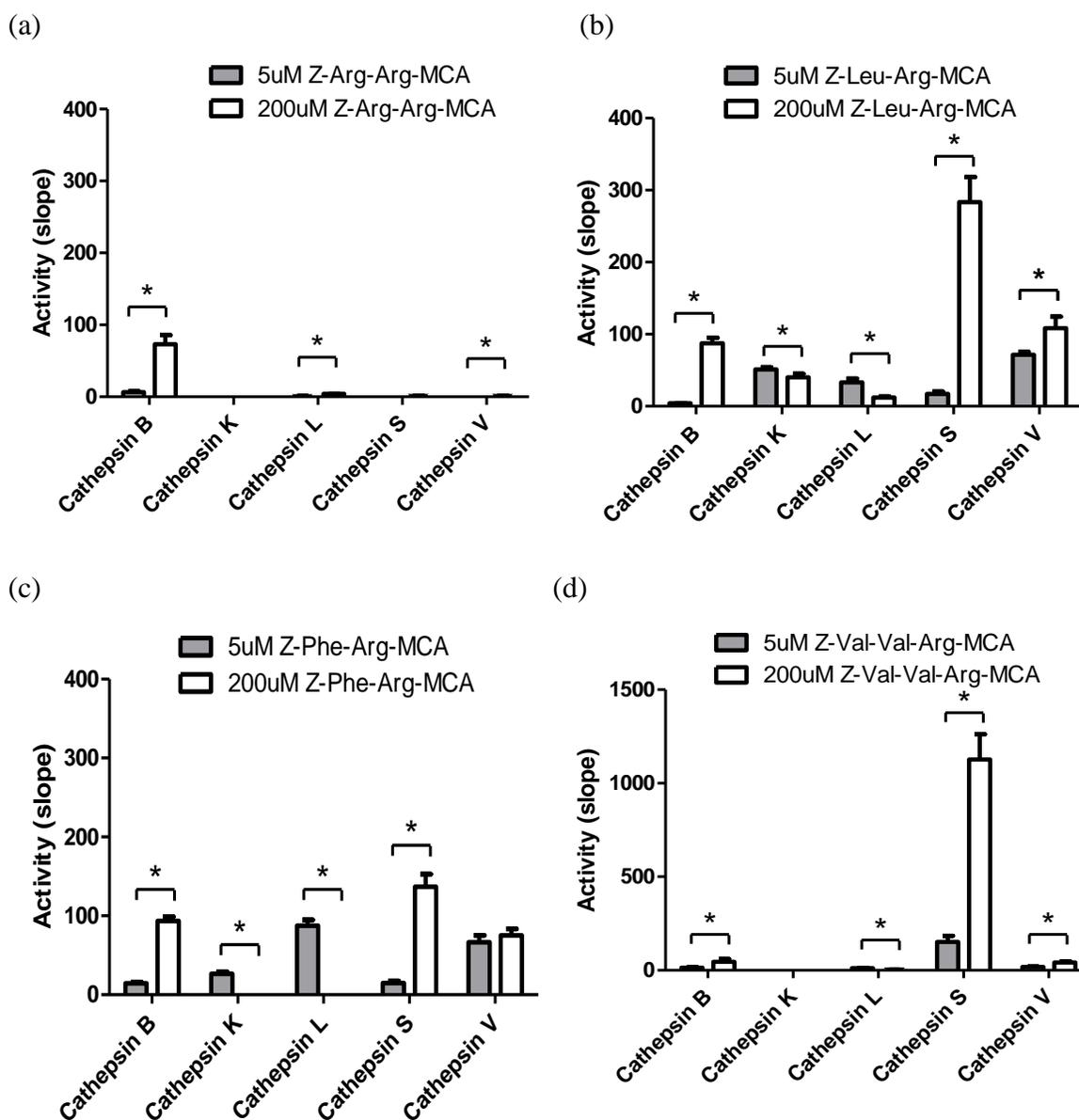


Figure 3.2.2 The Enzymatic Activities of Purified Cathepsins B, K, L, S, and V were Compared Using 4 Different Fluorogenic Substrates at 5 μM and 200 μM. (a) 5 μM and 200 μM Z-RR-MCA, (b) 5 μM and 200 μM Z-LR-MCA, (c) 5 μM and 200 μM Z-FR-MCA, (d) 5 μM and 200 μM Z-VVR-MCA. The pH of activity buffer was kept at 5.5 in order to measure the optimal activities of recombinant cathepsins. Error bars represent mean ± SD from three independent experiments (*p < 0.05).

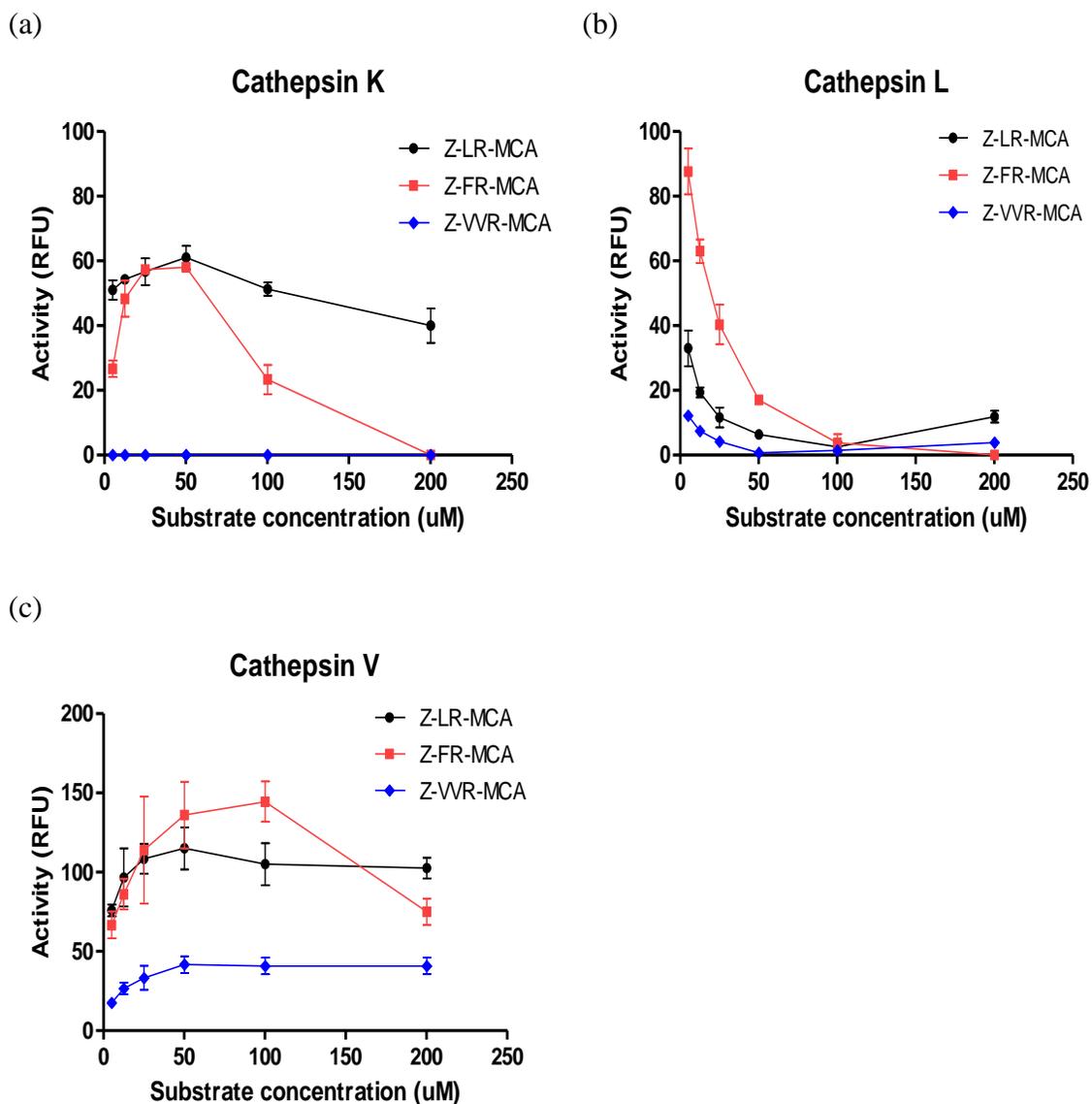


Figure 3.2.3 The Enzymatic Activities of Purified Cathepsins K (5 nM), L (5 nM), and V (5 nM) were Compared Using 3 Different Fluorogenic Substrates at Different Concentrations. (a) Z-LR-MCA, (b) Z-FR-MCA, (c) Z-VVR-MCA. The pH of activity buffer was kept at 5.5. Error bars represent mean \pm SD from three independent experiments (* $p < 0.05$).

3.2.3 Cathepsin Activities Expressed in Macrophage Cell Extracts

After evaluating the specificity of cathepsin substrates with recombinant enzymes, I attempted to test the feasibility of those assays in biological samples as designed by the assay providers. The activity of cathepsins within macrophage cell extracts was measured using four peptidyl substrates with/without protease inhibitors. The cell extracts were incubated with 5 μ M of 4 peptidyl-MCA substrates: Z-RR-MCA, Z-LR-MCA, Z-FR-MCA, and Z-VVR-MCA. 5 different inhibitors including E64, CatSI, CA074, GM6001, and 3,4-DCIC were used in order to characterize the contribution of each cathepsin towards substrate hydrolysis. The cell extracts were incubated at pH 5.5 for 1 hr to allow optimal activity conditions for cathepsins.

The cathepsin B specific substrate, Z-RR-MCA, was introduced to the cell extracts with 5 protease inhibitors. E64 was used in order to inhibit all cathepsin activities present in the cell extracts. As shown in figure 3.2.4a, E64 was capable of inhibiting ~95% of the enzymatic activities expressed in the cell extracts. CatSI is a potent and selective inhibitor of cathepsin S. With CatSI, the hydrolysis of Z-RR-MCA was surprisingly reduced by approximately 50%. As Z-RR-MCA is a sufficiently specific substrate for cathepsin B and only negligibly degraded by cathepsin S, it is assumed that under the assay condition (1 hour incubation time) CatSI at least partially inhibits cathepsin B activity. CatSI reacts with nitrile compound covalently, which has only a two order of magnitude higher specificity for cathepsin S over cathepsin B but more than a 3 order of magnitude higher affinity when compared with cathepsins K and L [59]. A selective irreversible cathepsin B

inhibitor, CA074, reduced the activity by ~95%. GM6001, a reversible matrix metalloproteinase (MMP) inhibitor and 3,4-DCIC, a potent irreversible serine protease inhibitor, showed no significant inhibition towards the hydrolysis of Z-RR-MCA. This indicates that the activity measured with the cathepsin B substrate is indeed cathepsin B.

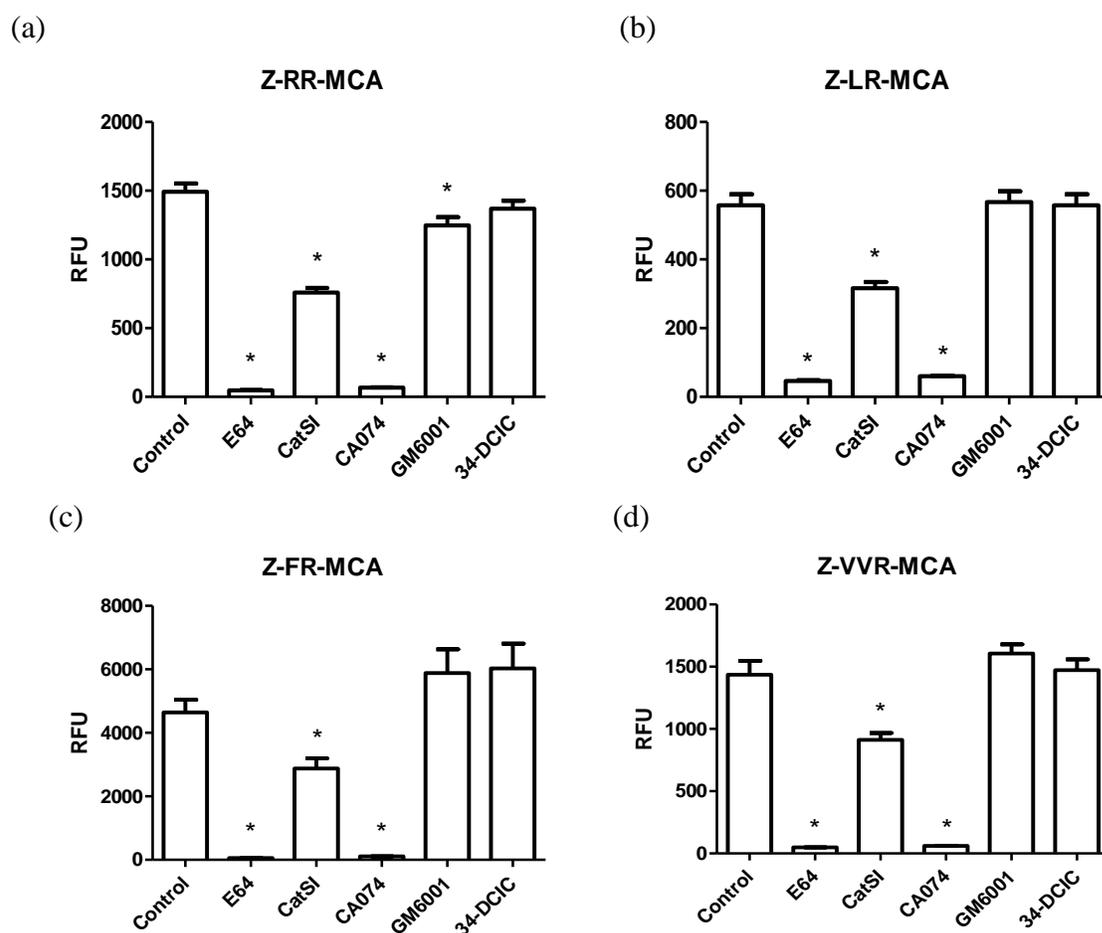


Figure 3.2.4 Measurement of Enzymatic Activities Expressed in RAW264.7 Cell Line Extracts Using 4 Different Substrates. (a) 5 μ M Z-RR-MCA, (b) 5 μ M Z-LR-MCA, (c) 5 μ M Z-FRMCA, (d) 5 μ M Z-VVR-MCA. The pH of activity buffer was kept at 5.5 in order to measure the optimal activities of cathepsins expressed in macrophages. Error bars represent mean \pm SD from three independent experiments.

The cathepsin K preferred substrate, Z-LR-MCA, revealed similar trends as Z-RR-MCA (Figure 3.2.4b). E64 and CA074 allowed 90% reduction of its enzymatic activity. With the cathepsin S inhibitor, there was a 45% decrease in substrate hydrolysis which may again indicate a partial cathepsin B inhibition. GM6001 and 3, 4-DCIC revealed no significant inhibition towards the hydrolysis of Z-LR-MCA.

Similar to the previous results, the cathepsin L preferred substrate (Z-FR-MCA) showed almost complete inhibition of enzymatic activities with 10 μ M E64 and 10 μ M CA074 (figure 3.2.4c). With CatSI, the activities were reduced by 40%, and GM6001 as well as 3,4-DCIC had negligible reduction in the hydrolysis of Z-FR-MCA.

The substrate, Z-VVR-MCA, also known as cathepsin S specific substrate, showed predictable results that were similar to the previous observations. As shown in figure 3.2.4d, E64 and CA074 completely inhibited the hydrolysis of this substrate. CatSI showed 40% reduction towards the substrate inhibition. The general MMP inhibitor, GM6001, and a serine protease inhibitor, 3,4-DCIC, had no significant effect of substrate hydrolysis.

In summary, the use of apparently specific cathepsin substrates does not allow the identification of individual cathepsin activities in cell extracts. When the substrate assays were complemented with selective or pan-cathepsin inhibitors, all four substrates revealed the same pattern. Instead of measuring individually cathepsins K, L, S, and B, the data imply that I only measured cathepsin B in the macrophage extracts. The pan-cathepsin inhibitor E64 and the cathepsin B-specific inhibitor CA074 inhibited the hydrolysis of all

four substrates equally and almost completely. This again underlines the limited suitability of these substrates to determine the activity of individual cathepsins.

3.2.4 Cathepsin Activities Measured Within Plasma and Serum Samples

Synthetic peptide substrates have been used to measure cathepsin activities in serum samples [75]. Here, I evaluated human blood plasma and serum samples for cathepsin activities and determined inhibitory effects of the samples on cathepsin activities. Increasing concentrations of plasma or serum were incubated with Z-FR-MCA (5 μ M) in reaction buffer (pH5.5). No enzymatic activity was observed (figure 3.2.5) in the samples under cathepsin suitable assay conditions. This indicates that there is no Z-FR-MCA hydrolysis activity present in the samples tested.

Plasma concentration	Slope	Serum concentration	Slope
0.5%	0	0.5%	0
2%	0	2%	0
10%	0	10%	0
20%	0	20%	0

Figure 3.2.5 Measurement of Cathepsin L Activity in Increasing Plasma/Serum Concentrations Using 5 μ M Z-FR-MCA. The pH of activity buffer was kept at 5.5 in order to measure the optimal activities of cathepsins.

Next, I tested if there are any natural inhibitors present within the plasma and serum preparations that may interfere with the measurement of cathepsin L activity. 10 μ L of purified cathepsins (5 μ M) and 10 μ L of blood plasma/serum were mixed, and 5 μ L of the

mixture was introduced into activity buffer (pH 5.5) with the addition of Z-FR-MCA. The measurement was taken at 3 different time points: 1 minute, 30 minutes, and 60 minutes. Active recombinant cathepsin B (2.5 μM) was mixed either in assay buffer alone or with plasma/serum in the activity buffer for 1 minute, 30 minutes, and 60 minutes. In figure 3.2.6a, the activity of cathepsin B was consistent throughout 3 time points in the assay buffer alone but dropped dramatically by 80% after only one minute incubation with a plasma or serum sample.

Similar quick inactivation of cathepsins K and L were observed when incubated with plasma/serum samples. In figure 3.2.6b and 3.2.6c, 2.5 μM cathepsin K and 2.5 μM cathepsin L were incubated with plasma or serum in the same manner as discussed for cathepsin B. As soon as the recombinant enzyme was introduced to blood plasma or serum samples, the peptidolytic activities of cathepsins K and L completely vanished, and absolutely no fluorogenic signal was measured at 3 different time points.

Lastly, in figure 3.2.6d, the peptidolytic activity of cathepsin V (2.5 μM) was determined. The activity of cathepsin V was severely reduced by 90~98% after the addition of blood plasma/serum at all three time points.

In summary, the activities of recombinant cathepsins was reduced significantly or completely abolished after introducing blood plasma or serum to the recombinant cathepsins. This suggested the inhibition of cathepsins by serum/plasma resident protease inhibitors and/or the unfavorable neutral pH in the samples.

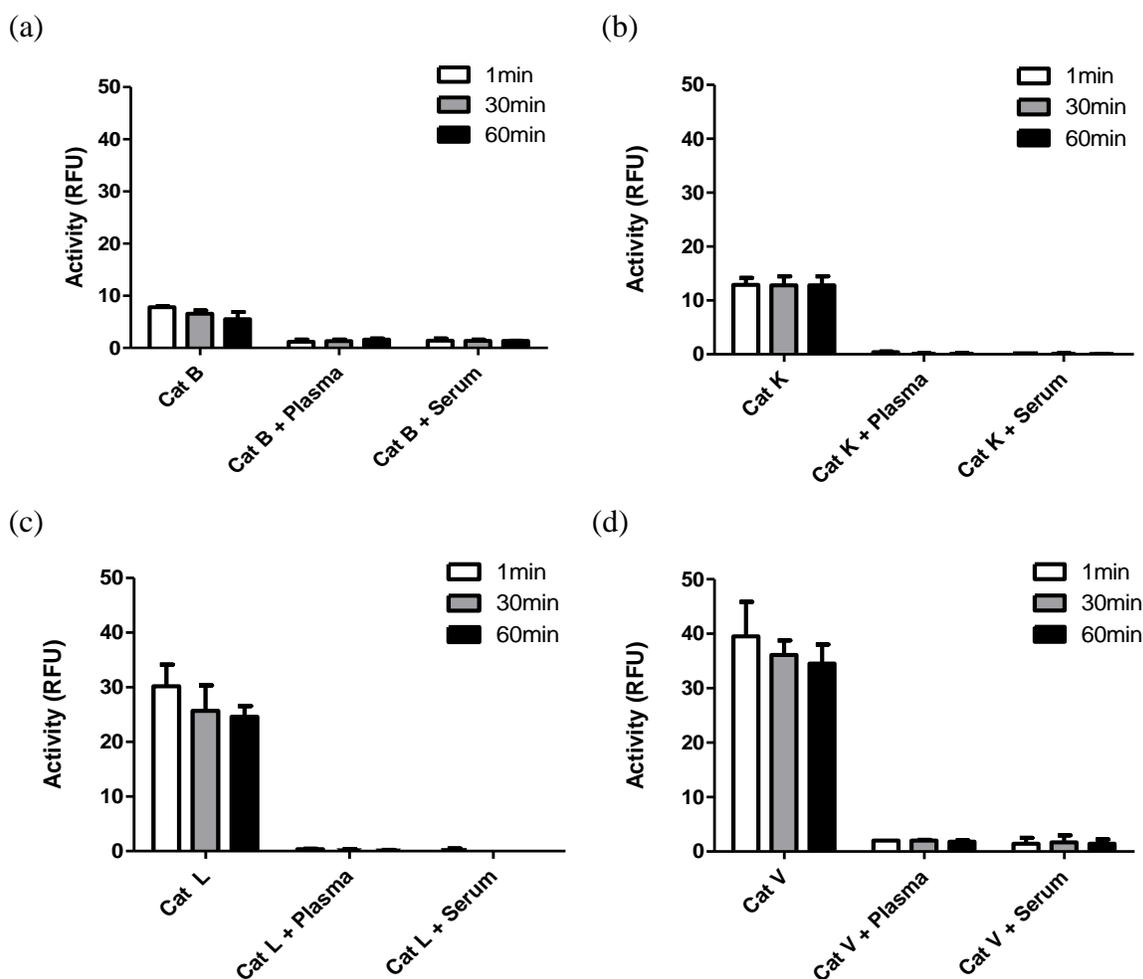


Figure 3.2.6 Measurement of Recombinant Cathepsins With or Without Plasma/Serum at 3 Different Time Points: 1 minute, 30 minutes, and 60 minutes using 5 μM Z-FR-MCA. (a) Recombinant cathepsin B (b) Recombinant cathepsin K with (c) Recombinant cathepsin L (d) Recombinant cathepsin V. Error bars represent mean ± SD from two independent experiment.

To eliminate the possibility that our substrates used (Z-peptidyl-MCA) compared to the commercial substrates of the assays kits (Ac-peptidyl-AFC) had potentially different substrate specificity features when applied to biological samples, I compared both

substrates side by side. Here, I measured the apparently cathepsin L mediated substrate cleaving activities in a cell extract of the Raw 264.7 cell line in the absence and presence of different protease inhibitors. As shown in figure 3.2.7, both substrates revealed identical activity profiles. Based on the inhibitor profiles both assays measured cathepsin B instead of cathepsin L. Thus, it can be concluded that both substrates do not reflect cathepsin L activities in the macrophages cell extracts and that probably also the other commercial substrates for cathepsin K and S of the AFC substrate class are unsuitable for the appropriate cathepsin determination as I have shown for the MCA substrates.

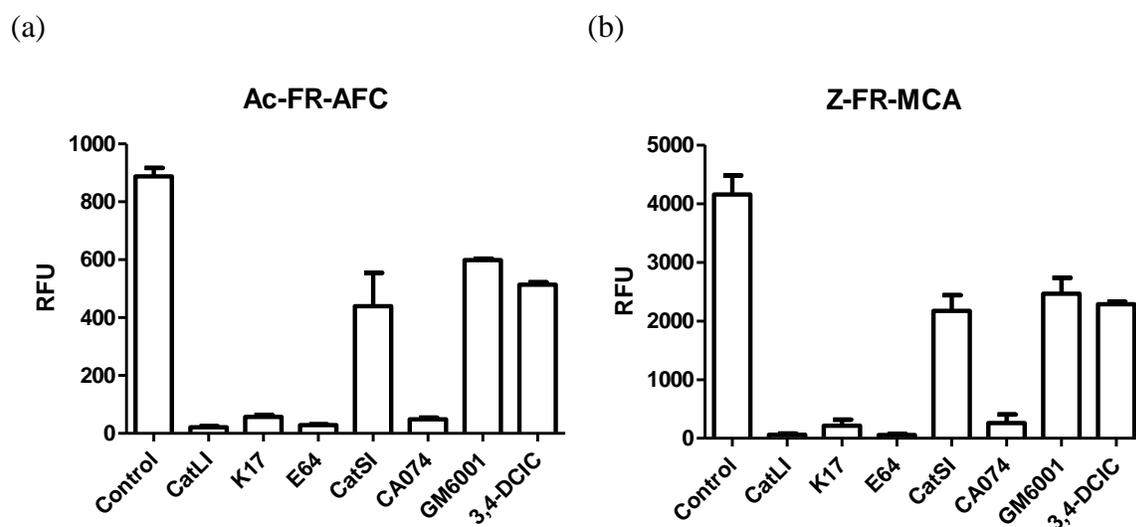


Figure 3.2.7 Measurement of Enzymatic Activities Expressed in RAW264.7 Cell Line Extracts Using 2 Different Substrates. (a) 5 μ M Ac-FR-AFC (b) 5 μ M Z-FR-MCA. The pH of activity buffer was kept at 5.5 in order to measure the optimal activities of recombinant cathepsins. Error bars represent mean \pm SD from two independent experiments.

4 DISCUSSION

4.1. Elastolytic Activities of Macrophages

4.1.1 Quantification of Elastolytic Activities of Cathepsins B, K, L and S Expressed in Mouse Peritoneal Macrophages.

Cathepsins expressed in macrophages have been recently implicated in atherosclerosis [66, 76], which is characterized by a continuous remodeling of the extracellular matrix in the artery wall. ECM is usually composed of fibrillar collagens, elastin, and proteoglycans. Various proteases including cathepsins [41], matrix metalloproteinases [77], and leukocyte elastase [38] are expressed in macrophages and are known to degrade ECM components including elastin [38, 76, 78].

In this study, focus has been given to characterizing the elastolytic activities of cathepsins B, K, L, and S expressed in macrophages. Elastin is an essential component of the ECM, and it is responsible for providing rigidity and elasticity to the blood vessels [79]. Therefore, elastin degradation within the blood vessel may cause instability of the vessel structure which can possibly lead to rupturing of the blood vessels and thrombosis. Furthermore, the gene expression and protein levels of elastolytic cathepsins were found to be increased in atherosclerotic lesions, indicating its significant involvement in pathological conditions [80, 81]. Elastolytic cathepsins were mostly found in macrophages of lipid-rich regions of

atherosclerotic plaque [42]. For these reasons, I was interested in characterizing the individual elastolytic cathepsins.

Using an *in vitro* elastin degradation assays, I determined the elastase activities of cathepsins B, K, L, and S expressed in murine macrophages (figure 3.1.1). In wild-type macrophages, total cathepsin activity contributed 40% towards elastin degradation as determined using the pan-cathepsin inhibitor K17. Out of this 40% of total cathepsin contribution, 10% were due to cathepsin B activity as CA074 yielded a 10% reduction of the total elastase activity. The cathepsin S specific inhibitor, CatSI did not inhibit the overall elastolytic activity in macrophages indicating that cathepsin S does not contribute to the elastin degradation. Thus, the remaining 30% were assumed to be due to cathepsin K and cathepsin L activities.

In cathepsin K^{-/-} macrophages, 40% of the overall elastolytic activity was assigned to cathepsins (K17 inhibitable activities). Each, cathepsin B (CA074 inhibitable) and cathepsin S (CSI inhibitable) contributed 10% towards elastolysis, and the remaining 20% was assumed to be due to cathepsin L. This was a surprising result; in wild-type macrophages, cathepsin S was not significantly involved in elastin degradation, but it contributed 10% towards elastolysis in cathepsin K^{-/-} macrophages. This may indicate an increase in cathepsin S expression and activity in cathepsin K deficient macrophages as a compensation for the loss of cathepsin K activity.

However, even with the compensatory mechanism of cathepsin S, the overall elastolytic

activity of cathepsin K-deficient macrophages was about 20% lower than in wild-type macrophages. It has been already established that the deficiency in cathepsin K leads to less breaks in elastic lamina of atherosclerotic lesions [44]. This implied that cathepsin K deficient macrophages were less efficient in elastin turnover than the wild-type macrophages even with the up-regulation of cathepsins S.

The increased expressions of compensating cathepsins in cathepsin deficient models were even more pronounced in cathepsin L^{-/-} macrophages. Surprisingly, the total cathepsin contribution towards DQ-elastin cleavage in cathepsin L^{-/-} macrophages was shown to be 80% (K17 inhibitable) when compared to the 40 % of the wild-type macrophages. This implies that there are significant elevated expressions of compensating cathepsins in cathepsin L^{-/-} macrophages. 40% of elastolysis were attributed to cathepsin B (CA074 inhibitable), and another 40% was due to cathepsin S activity (CatSI inhibitable). For comparison, cathepsin B only has contributed ~10% in wild-type and cathepsin K^{-/-} macrophages. Similarly, cathepsin S did not contribute towards elastin degradation in wild-type macrophages, and has only slightly increased to ~10% in cathepsin K^{-/-} macrophages. In contrast, cathepsin L^{-/-} macrophages revealed a 40% contribution of cathepsin S towards elastin degradation.

The compensatory effect towards cathepsin deficiencies has been documented by others. Sevenich and colleagues demonstrated that the role of cathepsin B was compensated by cathepsin Z in cathepsin B^{-/-} mice [82]. Moreover, cathepsin L was up-regulated in thyroids of cathepsin K^{-/-} mice [83]. Therefore, in our studies, it can be speculated that the

expressions of cathepsin B and cathepsin S have significantly increased in cathepsin L^{-/-} macrophages to overcome the lack of cathepsin L. Therefore, there was no difference in the elastolytic activities of wild-type and cathepsin L deficient macrophages. This however was in contrast with a study done by Kitamoto *et al* with cathepsin L deficient mice [84]. Their group showed that the cathepsin L-null mice had significantly less atherosclerotic lesions and reduced levels of collagen and medial elastin degradation. This contrasting result suggests that the degradation of insoluble elastic fibres *in vivo* may be different from the degradation mechanism of soluble DQ-elastin. Kitamoto's study was performed in atherosclerotic conditions where the mice were deficient in LDL receptor and they were fed with western diet for 12 and 26 weeks. In this case, the lesions are composed of macrophages, SMCs, lipid cores, fibrous cap, elastin degradation products, and cytokines, which may be involved in complicated mechanisms of up- or down-regulation of proteases and other signaling molecules. Also, smooth muscle cells have been described as major source of cathepsin K and S expression in atherosclerotic lesions and in aneurysms [85-88]. In the DQ-elastin degradation assay, the elastin degradation was done by only macrophages, and other atherosclerotic conditions were not present; there were no SMCs, cytokines or elastin degradation products that may have been involved in signaling pathways for up- or down-regulation of cathepsins. This may suggest that the observed differences in elastin degradation in intact plaques and isolated macrophages were due to these other factors and in particular due to cathepsin L-expressing SMCs.

4.1.2 Quantification of Elastolytic Activities of Intra- and Extracellular Cathepsins Expressed in Mouse Macrophages

For intracellular degradation of elastin fibres, macrophages are known to uptake extracellular material by phagocytosis [89]. In order to engulf elastin fragments, highly cross-linked elastin fibres within the extracellular matrix must be initially degraded into “phagocytosable” fragments by MMP-12, leukocyte elastases, and/or cathepsins [38, 90]. In the extracellular degradation pathway, cathepsins are secreted into an acidified microenvironment in order to destruct the water-insoluble elastin fibres. Firstly, macrophages are required to bind tightly onto the extracellular elastin and to express high levels of macrophage vacuolar-type H⁺-ATPases. This leads to the generation of an acidic extracellular environment where cathepsins are secreted to perform maximal activity in elastin degradation [91].

In figure 3.1.4, I have demonstrated the intracellular uptake of DQ-elastin by macrophages. The phagocytosed DQ-elastin was detected by visualizing the green fluorescence signals, which was localized adjacent to the nuclei (Blue – DAPI staining). This confirmed the assumption that DQ-elastin is internalized by macrophages for intracellular lysosomal degradation. To discriminate between extra and intracellular elastin degradation, I evaluated the effects of a pan cathepsin and a cathepsin B specific inhibitor in their membrane permeable and non-permeable versions. Both, intra- and extracellular inhibitor applications, revealed a similar intracellular fluorescence indicating no difference between the two inhibitor types. It should be also noted that this method was not suited to determine an

overall inhibitory effect on DQ-elastin degradation as other proteases continued to degrade this substrate and still generated a strong intracellular fluorescence. However, the experiment clearly demonstrated the intracellular degradation of DQ-elastin.

In wild-type, cathepsin K^{-/-}, and Raw264.7 macrophages, it was shown that the extracellular (E64) and intracellular (E64d) cathepsin-mediated elastase activity is 20~30% each (figure 3.1.2). E64 treated macrophages were thought to reveal the contribution of extracellular activity of elastolytic cathepsins as E64 is an inhibitor that does not penetrate cells. However, it has been established already that macrophages are capable of performing endocytosis and it is likely that E64 together with DQ-elastin has been taken up by macrophages through pinocytosis, and eventually inhibits intracellular cathepsin activity [66]. Thus, E64 is assumed to act as extra- and intracellular cathepsin inhibitor. Despite the fact that E64 inhibits both extra- and intracellular cathepsins, E64 most likely measured the intracellular elastin turnover rather than the extracellular degradation. Usually, the macrophages are required to be attached to the extracellular insoluble-elastin in order to create an acidic milieu which creates a proper environment for cathepsins to be secreted and perform enzymatic reactions [91]. However, in our experimental studies, the proper acidic environment was not provided with the soluble DQ-elastin, and cathepsins were likely unable to perform extracellular elastin degradation. Therefore, it can be concluded that most of the elastolysis took place intracellularly after the internalization of DQ-elastin, and the 20~30% reduction with E64 is assumed to be due to the contribution of intracellular cathepsin activities as E64 was co-pinocytosed with the DQ-elastin. On the other hand, E64d has been characterized as an intracellular inhibitor; E64d is an ester form of E64 and

is only active upon cleavage by endogenous esterases [92]. E64d allowed me to quantify the elastolytic activities of intracellular cathepsins only. E64d was able to enter the cell by moving across the cell membrane as well as by pinocytosis. E64d treatment revealed 20~30% of intracellular cathepsin activity towards elastin degradation. In cathepsin L^{-/-} macrophages, E64 and E64d both revealed ~60% reduction in elastin degradation, which represented mostly intracellular elastolytic activities of cathepsins due to the same explanation provided above.

CA074 (non-cell permeable) and CA074-Ome (cell permeable) inhibitors were also applied to macrophages in order to measure the extra- and intracellular cathepsin B activities in elastin degradation (figure 3.1.2). Similar to the experiments with the E64 derivatives, the elastin degradation was mostly assumed to be due to intracellular cathepsin B activity. As CA074 and CA074-Ome showed a similar efficiency in inhibiting DQ-elastin degradation, it is tempting to speculate that CA074 primarily acts intracellularly after pinocytosis. In wild-type and cathepsin K^{-/-} macrophages, there was a ~10% contribution of cathepsin B towards elastin degradation, which is thought to be due to elastolytic activity of intracellular cathepsin B. In cathepsin L^{-/-} macrophages, 30~40% of elastolytic activity was a result of intracellular cathepsin B activity. There was also an increased contribution of cathepsin B towards elastin degradation in cathepsin L^{-/-} macrophages when compared to the wild-type or cathepsin K^{-/-} macrophages. It can be assumed that the expression level of cathepsin B was significantly elevated in cathepsin L^{-/-} macrophages in order to compensate for the loss the cathepsin L activity. Raw264.7 macrophages revealed no significant reduction in elastin degradation with CA074 and CA074-Ome. This implied that the Raw264.7 macrophage cell line does not express intracellular cathepsin B that is enrolled in

elastin degradation. Figure 4.1.1 summarizes the intracellular effects of "extracellular" inhibitors.

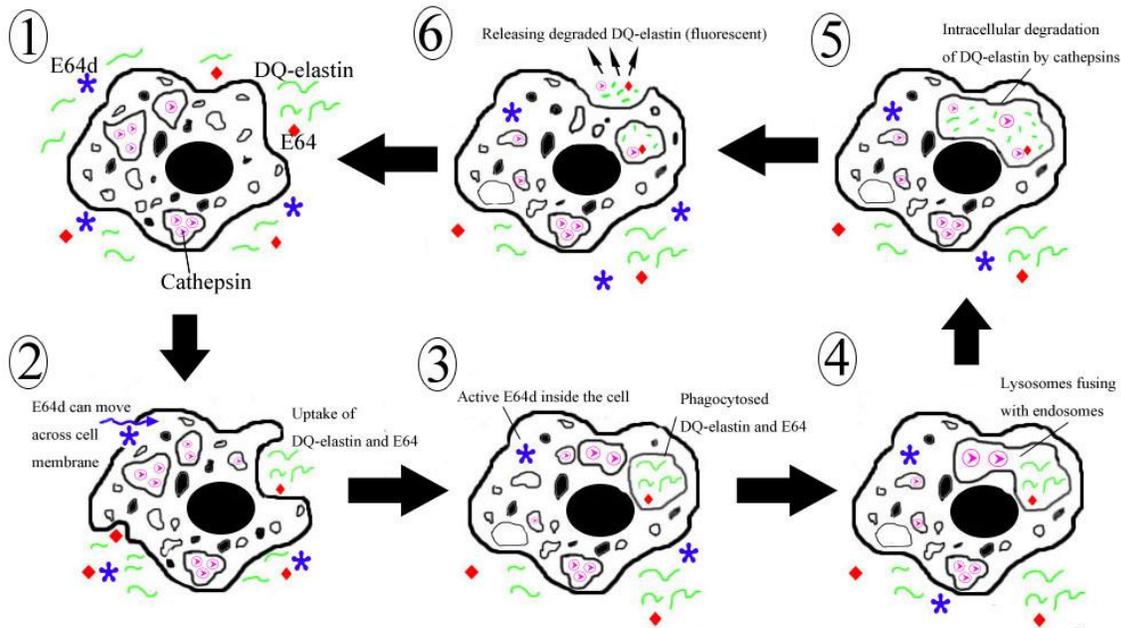


Figure 4.1.1 Overview of Intracellular Degradation of DQ-elastin by Cathepsins Expressed in Macrophages and the Effect of Extra- and Intracellular Inhibitors (E64 and E64d). (1) Macrophage is surrounded by DQ-elastin and inhibitors (2) Macrophage can uptake DQ-elastin and E64 (extracellular cathepsin inhibitor). E64d is an intracellular cathepsin inhibitor, which permeates the cell membrane. (3) Phago/pinocytosed DQ-elastin and E64 are localized in the endosome. (4) Endosome is fused with lysosomes containing cathepsins and other proteases that are able to degrade DQ-elastin. (5) Intracellular degradation of DQ-elastin is observed, and E64 may inhibit cathepsin activity. (6) Intracellularly degraded DQ-elastin is exocytosed, and the fluorescent DQ-elastin fragments are released for fluorescence measurement.

4.1.3 Quantification of Elastolytic Activities of Non-cysteine Proteases Expressed in Mouse Macrophages

The contribution of non-cysteine proteases towards elastin degradation were tested using the general MMP inhibitor GM6001 and the general serine protease inhibitor, 3,4-DCIC (figure 3.1.3).

In the wild-type and cathepsin K^{-/-} macrophages, GM6001 was used in order to suppress the total activity of MMPs in elastin degradation. The experiments revealed a contribution of ~10% of the total elastolytic activity by MMPs. In contrast, cathepsin L^{-/-} and Raw264.7 macrophages did not show a significant contribution from MMPs to elastinolysis. These data indicate that MMPs have little yet significant roles in elastin degradation in wild-type and cathepsin K^{-/-} macrophages, and no critical role in cathepsin L^{-/-} and Raw264.7 macrophages. Despite the widely accepted role of MMPs in degrading insoluble elastin fibers [93], these results revealed that MMPs only contribute little or nothing to the degradation of elastin. Here, soluble DQ-elastin substrates are taken up by macrophages and digested intracellularly. However, MMPs are not involved in intracellular degradation; rather they are zinc-dependent enzymes that are enrolled in the degradation of extracellular matrix [94, 95].

A similar scenario was shown with the treatment of 3,4-DCIC, which was used to characterize the contributions of serine proteases towards elastin degradation. In wild-type, cathepsin K^{-/-}, and Raw264.7 macrophages, the 3,4-DCIC reduced the elastolytic activity

by ~20%. It can be assumed that the ~20% elastolytic activity is due to serine proteases expressed in macrophages. It has been known that there are leukocyte elastases and cathepsin G in macrophages [96], which are speculated to be involved in the turnover of elastin and other matrix components. Cathepsin L deficient macrophages did not show any inhibition with the use of 3,4-DCIC. This implied that serine proteases expressed in cathepsin L^{-/-} macrophages are not involved in elastin degradation. It was speculated that the expression levels of serine proteases are altered in cathepsin L^{-/-} macrophages reflecting their negligible role in elastolysis.

The combined inhibitor treatment (K17+GM6001+3,4-DCIC) in wild-type, cathepsin K^{-/-}, and cathepsin L^{-/-} macrophages reduced the elastolytic activity by 60~80% and it was not able to inhibit 100% of the elastolytic activity. This implied that there are other proteases that are involved in elastin degradation other than cathepsins, MMPs, and serine proteases. It can be speculated that the elastin degradation may be due to aspartic proteases [97], but further investigations are mandatory in order to confirm the role of aspartic proteases in elastin cleavage.

4.1.4 Cathepsins are Involved in Fibrinolysis

Atherosclerotic plaque is made up of various components such as macrophage foam cells, lipid-rich cores, elastin, collagen, fibrin and fibrous cap [29]. The fibrin clots are usually degraded by plasmin and other enzymes including cathepsin G releasing fibrin degradation products (FDP) [50]. The presence of fibrin and FDPs were known to contribute to

complication of the lesions by promoting cell proliferation, migration, and adhesion [30]. Moreover, it has been found that macrophage and SMCs express high levels of cathepsins which are potential candidates for degrading fibrin clots. Thus, we were interested in characterizing the fibrinolytic activities of cathepsins B, K, L, and V in comparison with plasmin.

The fibrinolytic activities of human recombinant cathepsins B, K, L, and V were relatively compared with plasmin to determine the fibrinolytic efficiency (figure 3.1.4). The half-lysis time for plasmin was ~100 minutes and plasmin was shown to be the most powerful enzyme in performing fibrinolysis. Cathepsin B was 4-fold less fibrinolytically active compared to plasmin. Cathepsins K and L were approximately 1.5-fold less active than plasmin in fibrinolysis. Cathepsin V was more efficient than cathepsin B in fibrin cleavage, but it was still 3-fold less efficient compared to plasmin.

Cathepsins B, K, L, and V seemed to be less active than plasmin in fibrinolysis, but they were definitely able to perform fibrinolytic activities with various efficacy. Thus, we speculate that the cathepsins are highly expressed in macrophages or SMCs in atherosclerotic plaques, and these cathepsins contribute to fibrin degradation promoting additional complications of the plaque. Future studies will be focused on the investigation of the macrophage-mediated fibrinolysis, and characterizing the contributions of each cathepsin towards fibrin degradation.

4.2. Characterization of Cathepsin-Specific Substrates

4.2.1 The Validity of Commercially Available Cathepsin Activity Assay Kits

Cathepsin cysteine proteases are critically involved in intracellular protein catabolism and extracellular matrix degradation. In my studies, focus was given to the enzymatic activities of cathepsins B, K, L, S and V. It is difficult to discriminate the contribution of an individual cathepsin in enzymatic processes. As shown in previous results, cathepsin knockout mice and selective inhibitors were used in order to quantify individual cathepsin activity towards elastin degradation (figure 3.1.1). On the other hand, cathepsin activity assay kits are commercially available and could be used to determine the contribution of individual cathepsins to the overall cysteine protease activities in macrophages and other biological samples. For example, studies from Timothy M. Cox and colleagues demonstrated the up-regulation of cathepsin B, K, and S activity in serum samples of patients with Gaucher disease before and after enzyme replacement therapy [75]. Another study done by Golovatch *et al.* showed increased cathepsin K activity in guinea pig lung extracts after smoke exposure [98]. Thus, I considered using these kits in quantifying individual cathepsin activities in cell extracts and blood serum in conjunction to their elastolytic activities.

The cathepsin B activity assay kit contained Ac-RR-AFC as a cathepsin B substrate, and cathepsin K activity assay contained Ac-LR-AFC as a cathepsin K specific substrate.

Cathepsin L and cathepsin S activity assay kits included Ac-FR-AFC and Ac-VVR-AFC, respectively. In my experimental studies, similar fluorogenic substrates, Z-XR-MCA, were used as an alternative. As an example, I have compared the activity of these two different substrates, Z-FR-MCA and Ac-FR-AFC. I have conducted identical activity assays with these two substrates, and compared if they generate any different results from what we have expected. I have used macrophage cell extracts with 5 different inhibitors. As shown in figure 3.2.7, these two substrates gave indistinguishable results and thus are interchangeable. Thus, my results obtained with the other Z-XR-MCA substrates are suitable to discuss the related commercial kits as well.

4.2.2 Cathepsin Specific Substrates Do Not Measure the Activity of Their Assigned Cathepsins, Rather the Substrates are Non-specifically Hydrolyzed by Many Cathepsins

I have performed several tests in order to establish the validity and reliability of these kits in measuring the activity of individual cathepsins. For example, the substrate, Z-FR-MCA, was assumed to be a cathepsin L specific substrate in the kit. However, our experimental data have disproved this assumption, and the substrate was hydrolyzed by other cathepsins such as cathepsins B, K, S, and V. Therefore, we have made thorough investigations on the validity of these kits.

Z-RR-MCA was the only substrate that was hydrolyzed by its assigned cathepsin which was cathepsin B. At 5 μ M substrate concentrations, substrates are below or at K_m

conditions for most cathepsins tested and thus allow an approximation of their specific activities (kcat/Km values). The cathepsin S preferred substrate (Z-VVR-MCA) showed the most sensitivity towards cathepsin S; however, cathepsins B and L were capable of hydrolyzing the substrate as well with only ~10% of cathepsin S activity. The cathepsin K and cathepsin L specific substrates were hydrolyzed by all five cathepsins with similar overall efficiency. For instance, the cathepsin K specific substrate, Z-LR-MCA, was hydrolyzed by all five cathepsins in an efficient manner, and instead of cathepsin K, cathepsin V possessed the most powerful enzymatic activity towards the substrate. The cathepsin L preferred substrate, Z-FR-MCA, was efficiently catalyzed by cathepsins L, S and V. These results were in contradiction with the claims stated on the kit protocols. Thus, extreme care must be taken in interpreting the results of these commercial kits.

I also investigated the importance of the substrate concentration, and its inhibitory effect on the activities of certain cathepsins (figure 3.2.2). A standard cathepsin activity assay is usually performed at 5 μM substrate concentration for the Km values for cathepsins are usually between 10~100 μM depending on the substrate and the individual protease. The assay kits recommend the usage of substrate concentration of 200 μM which would be above the Km values of most substrates for cathepsins and also would prevent the depletion of the substrate under the recommended assay conditions.

With the kit recommended substrate concentration (200 μM), cathepsin B showed the maximal hydrolase activity towards Z-RR-MCA (figure 3.2.2a), which was in agreement with the claims of the kit (Km for cathepsin B: 470 μM). Moreover, cathepsin S showed the

highest activity (figure 3.2.2d) towards 200 μM Z-VVR-MCA ($K_m = 18 \mu\text{M}$) and it was valid to be acknowledged as a cathepsin S substrate. However, problematic results were observed with cathepsin K and cathepsin L specific substrates, Z-LR-MCA and Z-FR-MCA (figure 3.2.2c and d). A significant substrate inhibition was observed at high substrate concentrations.

The kinetics of substrate hydrolysis showed a significantly decreased reaction rate at high substrate concentrations (figure 3.2.3), which differed from the simple Michaelis-Menten kinetics. This can be described in terms of substrate inhibition. The K_m value for cathepsin K towards Z-LR-MCA is 4 μM (Table 3.2.1). Therefore, with 5 μM Z-LR-MCA, the reaction rate of cathepsin K can be said to be at ~50% of its maximal velocity ($K_m = 1/2V_{\text{max}}$). At 50 μM substrate concentration, the maximum reaction rate is thought to be achieved ($V_{\text{max}} = 10K_m$). With Z-LR-MCA, the maximum reaction rate was observed with 50 μM substrate concentration (figure 3.7a). At the substrate concentration of 5 μM , the rate of hydrolysis was 80% of V_{max} . However, once the substrate concentration exceeded 50 μM , the activity started to drop and showed significant substrate inhibition. At 100 μM and 200 μM Z-LR-MCA, the activity was reduced to 80% and 60%, respectively. With Z-FR-MCA ($K_m = 7.4 \mu\text{M}$), a more significant product/substrate inhibition was shown at high substrate concentrations. At 5 μM Z-FR-MCA, the optimal rate of hydrolysis was observed at 50 μM , and at 5 μM , the activity was ~50% of V_{max} . Substrate inhibition was clearly shown above 50 μM substrate concentration; the activity was reduced by ~50% at 100 μM Z-FR-MCA, and eventually the enzyme was no longer active at 200 μM Z-FR-MCA. For cathepsin L, the K_m values for Z-LR-MCA and Z-FR-MCA are 13 μM and 0.8

μM , respectively. As shown in figure 3.2.3b, the maximal activities were shown at 5 μM substrate concentration. As the substrate concentration increased from 5 μM to 50 μM , the rate of hydrolysis decreased by 80~90%. Above the substrate concentration of 100 μM , cathepsin L no longer carried enzymatic activity. Here, cathepsins K and L clearly showed substrate or product inhibition at high substrate concentrations. Substrate inhibition occurs when enzymes are assumed to have two substrate binding sites, where at low substrate concentration, the high affinity binding site is occupied, and at high substrate concentration, the low affinity binding site is occupied which leads to decrease in the rate of production of the product [99]. Product inhibition is when the cleaved products are competing with the substrates to bind to the decreased activities at high substrate concentrations for cathepsins K and L. Further investigations are necessary in order to distinguish between substrate and product inhibition [100]. Consequently, cathepsin K and cathepsin L revealed little to no enzymatic activities at the excessive substrate concentration due to substrate/product inhibition. These results contradicted the proposed assay conditions of the assay kits manuals (recommended substrate concentration of 200 μM).

4.2.3 Cathepsin Activity Assay Kits Do Not Measure the Activity of Its Assigned Cathepsin in Biological Samples such as Cell Extracts

We have tested the cathepsin activity assay kits with cell extracts and blood plasma/serum in order to identify the validity of these kits towards measuring their assigned cathepsin activities.

Raw264.7 macrophages cell extracts were used in order to characterize 4 substrates towards biological samples (figure 3.2.4). For example, the cathepsin L substrate (Z-FR-MCA) was provided in the kit in order to measure only cathepsin L activity in cell extracts and biological fluids. In our previous experiments, the cathepsin L substrate (Z-FR-MCA) behaved in such a way that it was hydrolyzed by any cathepsins including cathepsins B, K, L, S, and V. Therefore, we wanted to explore further about the selectivity and validity of the substrates provided by the kit. The pH of activity buffer was kept at 5.5 and reducing buffer conditions (DTT) were used in order to allow for optimal conditions for the measurement of cathepsins expressed in macrophages

Firstly, the cathepsin B substrate, Z-RR-MCA, was relatively specific for cathepsin B, which CA074 reduced ~95% of the hydrolysis rate (figure 3.2.4a). As described in the results section, the 40% decrease with CatSI was assumed to be partially cathepsin B inhibition rather than cathepsin S inhibition. CatSI reacts covalently with nitrile compounds, which has only two orders of magnitude higher specificity for cathepsin S over cathepsin B, but more than a 3 order of magnitude higher affinities when compared with cathepsins K and L. Thus, it was concluded that the measured activity was mostly due to cathepsin B activity, and the substrate Z-RR-MCA is an adequate substrate to use for the measurement of cathepsin B activity. Surprisingly, a similar result was observed with the cathepsin K substrate. Z-LR-MCA was hydrolyzed by the macrophage cell extract (figure 3.2.4b) but the activity was also inhibited by CA074. This means that the recommended kit assay protocol was not measuring cathepsin K activity as claimed; rather the activity measured with the cathepsin K substrate was again cathepsin B. Thirdly, the substrate, Z-FR-MCA

(supposedly cathepsin L specific), was also hydrolyzed by cell extracts, but the activity was significantly reduced by CA074 and E64. The 45% decreased activity with cathepsin S inhibitor may again indicate a partial inhibition of cathepsin B (figure 3.2.4c). This implied again that the signal was due to cathepsin B activity rather than cathepsin L activity. Lastly, similar to previous investigations, the substrate, Z-VVR-MCA, was efficiently hydrolyzed by the cell extracts (figure 3.2.4d). However, the hydrolysis was completely inhibited using the cathepsin B inhibitor (CA074), and partially with cathepsin S inhibitor, CatSI. Consequently, the only measurable activities in cell extracts were found to be cathepsin B and the proposed detection of cathepsin S stated by the kit was proved to be false in this case.

Consequently, the substrates for cathepsin B, K, L, and S measured the activity of cathepsin B in macrophages cell extracts. The reasoning behind these results is that there are natural inhibitors expressed in biological samples, which may interfere with the measurement of cathepsin activity. Furthermore, the activities of these endogenous cysteine protease inhibitors such as the cystatins are extremely high towards most cathepsins with the exception of cathepsin B. K_i values are in the picomolar range (Table 1.2). Our assumption is that the activity of cathepsins K and L is most likely to be completely inhibited by the actions of cystatins, and the activity of cathepsin B is the only surviving activity due to its relative weak affinity towards cystatins. Therefore, it can be stated that the cathepsin activity assay kits does not measure its assigned cathepsin activity; rather the activity measured within cell extracts is mostly cathepsin B activity.

4.2.4 Cathepsin Activity Assay Kit Do Not Measure the Activity of Its Assigned Cathepsin in Biological Samples Such as Blood Plasma/Serum

We have also made an attempt to measure cathepsin activity in blood plasma/serum as has been claimed by others [75]. I have selected the cathepsin L substrate, Z-FR-MCA, to measure the activity of cathepsin L in blood plasma/serum. With increasing concentrations of plasma/serum at pH 5.5, I was not able to measure any activity (figure 3.2.5) which could be due to the known high concentration of cystatin C in serum and other biological fluids [101]. In order to test the presence of inhibitors within plasma/serum, I conducted an experiment where I added recombinant cathepsins to plasma/serum samples. Interestingly, recombinant purified cathepsins B, K, L, and V lost immediately 90~98% of their activity once they were added to the plasma/serum (figure 3.2.6). The result strongly supported the presence of high levels of cystatins in the biological samples.

Another reason for the loss of cathepsin activity in blood plasma/serum can be attributed to the neutral pH value. Cathepsins are known to be optimally active at an acidic pH (pH 5.5) with the exception of cathepsin S [19], whereas the blood plasma/serum or bodily fluids are in the range of neutral to slightly alkaline pH (pH 7.4). Therefore, the neutral pH of plasma may not serve as an adequate environment for cathepsins to be enzymatically active [102].

In conclusion, the commercially available cathepsin activity assay kits do not measure the true activity of its assigned cathepsin. The measurement of cathepsin activity in biological samples is not only affected by the natural inhibitors expressed in the samples, but also

affected by the pH conditions. These factors definitely interfere with the capability of these kits to measure cathepsin activity and they are to be used with great caution. When utilizing these kits, proper substrate concentrations and adequate use of inhibitors should be taken into an account in order to avoid the generation of erroneous results. Thus, it is likely that certain results in published papers [75, 98, 103, 104], which have used these kits, are questionable and their conclusions should be treated with caution.

5 CONCLUSION AND FUTURE DIRECTIONS

Because of their critical role in ECM remodeling, cathepsins have been considered as valuable therapeutic targets. In this study, the contribution of individual cathepsins towards elastin degradation was characterized, and the effect of cathepsin deficiency on the expressions of compensatory cathepsins has been evaluated. The identification of individual cathepsins that are enrolled in elastin degradation can promote the development of selective inhibitors that may benefit the treatment of atherosclerosis by slowing down the process of ECM remodeling. Further investigations should be focused on the quantification of up-regulated cathepsins in cathepsin deficient mice, and their effect in altering the elastin degrading mechanism via cytokines and inhibitors. Considering the rather dramatic effect of cathepsin L deficiency on the up-regulation of other cathepsins and the proposed functions of cathepsin L within the nucleus it would be interesting to study the potential role of cathepsin L in transcription factor/suppressor regulation.

In the second part of my research, commercially available cathepsin activity assay kits were evaluated for their validity and reliability. Experimental results suggest that most substrates used in the kits were not selective towards individual cathepsins. Also, the kits were unable to perform proper measurement of cathepsins in biological samples such as cell extracts or blood plasma/serum samples. Thus, the use of sufficient controls, such as panels of inhibitors, was highly recommended to validate or invalidate the predicted outcome of those assays. Future studies should be focused on developing improved substrates and methods to measure individual cathepsin activity within biological samples.

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