Review

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Cathepsin S: therapeutic, diagnostic, and prognostic potential

Abstract: Cathepsin S is a member of the cysteine cathepsin protease family. It is a lysosomal protease which can promote degradation of damaged or unwanted proteins in the endo-lysosomal pathway. Additionally, it has more specific roles such as MHC class II antigen presentation, where it is important in the degradation of the invariant chain. Unsurprisingly, mis-regulation has implicated cathepsin S in a variety of pathological processes including arthritis, cancer, and cardiovascular disease, where it becomes secreted and can act on extracellular substrates. In comparison to many other cysteine cathepsin family members, cathepsin S has uniquely restricted tissue expression and is more stable at a neutral pH, which supports its involvement and importance in localised disease microenvironments. In this review, we examine the known involvement of cathepsin S in disease, particularly with respect to recent work indicating its role in mediating pain, diabetes, and cystic fibrosis. We provide an overview of current literature with regards cathepsin S as a therapeutic target, as well as its role and potential as a predictive diagnostic and/or prognostic marker in these diseases.

Keywords: biomarker; inhibitor; probes; substrates.

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Introduction

Proteases are defined as enzymes that have the ability to degrade proteins through hydrolysis of peptide bonds which covalently link amino acids. Proteases make up approximately 2.8% of the protein coding human genome and are commonly involved in degradation pathways; removing proteins which may be unwanted or damaged (Puente et al., 2005; Rawlings et al., 2012). Fundamentally however, proteases are not simply limited to protein turnover, but also contribute to a wide range of physiological processes including cell signalling, control of receptor activation, chemokine/cytokine processing and antigen presentation (Turk et al., 2012a; Repnik et al., 2015). Given that proteolysis is generally an irreversible process, points in biological processes/pathways that are mediated by proteases are necessarily under tight regulation. Proteases can be regulated in a number of ways including compartmentalisation, post-translational modifications and the presence of anti-proteases (Brix et al., 2013). Unsurprisingly, loss of regulation can result in disruptive consequences for cellular and tissue environments manifesting in pathological roles such as aberrant signalling, manipulation of cytokine expression, neoangiogenesis, tissue remodelling, migration and apoptosis (Turk et al., 2004). As such, many proteases are considered therapeutic targets in a range of conditions including cancer, autoimmune, cardiovascular and neurodegenerative disease (Turk, 2006).

However, this area of drug development is not without its scars. In the early 1990s, matrix metalloproteinases (MMPs) were uncovered to hold potential as targets for the treatment of cancer and arthritis (Klein and Bischoff, 2011). However, this initial early promise soon gave way to disappointing results in clinical trials. MMP inhibition failed to live up to expectations due to inadequate understanding of MMP biology at this time, as several MMP homologues were subsequently identified, each with their own physiological role. As a result, the broad-spectrum inhibitors developed against MMPs resulted in a wide range of side effects and interest in inhibiting proteases that were not

fully characterised waned (Overall and López-Otín, 2002). As with many other types of potential enzymatic targets, these lessons demonstrate the importance in understanding and validating protease function in both normal and pathological processes in order to properly understand their therapeutic usefulness.

Cysteine cathepsins

The discovery of lysosomal cysteine proteases in the latter half of the 20th century originates in the isolation of the protease papain from the papaya fruit *Carica papya*, leading to the identification of the first cathepsin family members (Drenth et al., 1968). They were thought to solely involve the bulk degradation of damaged or unwanted proteins (Kominami et al., 1991; Saftig et al., 1995); however, in subsequent years alternative roles for individual cathepsins elucidated more unique functions such as bone resorption, protein processing, and antigen presentation (Turk et al., 2000).

The cathepsins are a diverse group of proteases expressed throughout the plant and animal kingdoms (Otto and Schirmeister, 1997). In humans there are fifteen cathepsins divided into three groups by their active site catalytic residue; serine (cathepsins A and G), aspartate (cathepsins D and E), and cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, W, and X). The cysteine cathepsins can be further divided by their proteolytic activity, with cathepsins S, K, V, F, and L showing endo-peptidase activity and cathepsins B, H, X, and C showing both endo- and exo-peptidase activity (Turk et al., 2012b).

Like many other proteases, the cysteine cathepsins are controlled in various manners, namely at the transcriptional, translational, and post-translational levels. Several transcription regulatory sites have been found in cathepsin genes including Sp1 and Sp3 binding sites which have been observed in cathepsin B, L, and S (Mohamed and Sloane, 2006). For example, the cathepsin S (*CTSS*) gene contains an interferon-stimulated response element (IRSE) within its promoter region and treatment of cells with IFN-γ can upregulate the protease species (Storm van's Gravesande et al., 2002).

At the protein level, cysteine cathepsins are synthesized as pre-proenzymes, with the presence of a propeptide domain that occludes the active site, rendering the protease inactive (Dickinson, 2002). A further level of control is conferred by compartmentalisation, where cathepsins are normally confined to the endo-lysosomal lumen which prevents active species inadvertently degrading host proteins and also helps maintain the stability of the protease in the acidic pH environment.

Once activated, cysteine cathepsins can be controlled through the presence of endogenous inhibitors known collectively as the cystatin family. The cystatin family of cysteine protease inhibitors may be divided into three sub-families; the stefins, kininogens, and cystatins. The cystatins are found ubiquitously expressed and are thought to be involved in the control and sequestering of unwanted cathepsin activity (Ochieng and Chaudhuri, 2010). The cystatin family inhibitors hold an important role in balancing proteolytic roles in normal physiology, and dys-regulation has been shown to play a role in the progression of disease pathology. This has been demonstrated in tumourigenesis, where cystatin levels were shown to be reduced in late stage tumours, and patients with higher cystatin C levels were shown to be more likely to suffer from chronic kidney disease and cardiovascular disease (Korolenko et al., 2003; Kothapalli et al., 2003; Taglieri et al., 2009).

Cathepsin S

Cathepsin S is unique amongst the cysteine cathepsin family due to restricted tissue expression, associated with antigen presenting cells localised in lymph and spleen, as well as other immune cells such as macrophages (Turnsek et al., 1975; Maciewicz and Etherington, 1988; Xin et al., 1992; Morton et al., 1995). Biochemically, cathepsin S is also different from many family members in its ability to retain activity at a neutral pH (Chapman et al., 1997). As such, these traits highlight cathepsin S as an ideal target for disease treatment, as with its highly restricted expression, therapeutic inhibition should minimise potential side effects. Furthermore, its enhanced stability at a neutral pH over other family members highlights its increased potential for involvement in extracellular proteolytic activities (Shi et al., 1994; Jordans et al., 2009).

Produced from its cognate *CTSS* gene on chromosome 1q21, human cathepsin S is synthesized as a pre-proenzyme of 331 amino acids in length, containing a signal domain, pro-peptide domain and mature domain of 16, 98, and 217 amino acids in length respectively (Lecaille et al., 2002). The nascent protein is translated at the rough endoplasmic reticulum, and it is thought from detailed folding studies that the propeptide inhibitory domain also acts as a chaperone, facilitating the correct folding of the preform as an inhibited complex in the ER lumen (Nissler et al., 2002).

Using murine models, it has been revealed that mice lacking the *Ctss* gene display diminished MHC class II (MHCII) antigen presentation (Nakagawa et al., 1999). MHCII antigen presentation requires the presence of the type II glycoprotein invariant chain (Ii; CD74), which is involved in both the folding and transport of the MHCII cassette to the endo-lysosomal pathway, and as a pro-form peptide stopping binding of self-antigens. Upon reaching the endo-lysosomal pathway in antigen presenting cells, the invariant chain is removed from the MHCII complex to allow subsequent antigen loading. The removal of the invariant protein is mediated by several discrete proteolytic cleavages, which includes cathepsin S (Riese et al., 1996; Honey and Rudensky, 2003).

Cathepsin S and disease

As with many other protease species, the association of cathepsin S with a range of substrates and pathological conditions are increasingly being made (Small et al., 2011) (Figure 1). As tools such as genetic ablation models, chemical biology probes and substrates are developed, the role of cathepsin S in these diseases is being more comprehensively dissected, highlighting its potential as a therapeutic target.

Autoimmune diseases

Autoimmune disease is characterised as the host's recognition of self-antigens as foreign entities, inducing a subsequent immune response against these self-antigens, resulting in an inappropriate inflammatory response. Strategies to prevent or retard the presentation of these self-antigens may have therapeutic benefits. Given that cathepsin S is a protein that is produced in response to inflammatory stimuli and that its genetic ablation can attenuate MHCII presentation, some interest has historically been focussed on the protease as a target in immunological disorders (Conus and Simon, 2010). Inhibitors of cathepsin S in autoimmune diseases such as lupus nephritis have proven successful in an *in vitro* setting, rationalizing research into cathepsin S as a target for other autoimmune diseases (Rupanagudi et al., 2015), including rheumatoid arthritis. Rheumatoid arthritis is a chronic pro-inflammatory autoimmune disease characterised by the destruction of bone and cartilage, which is facilitated

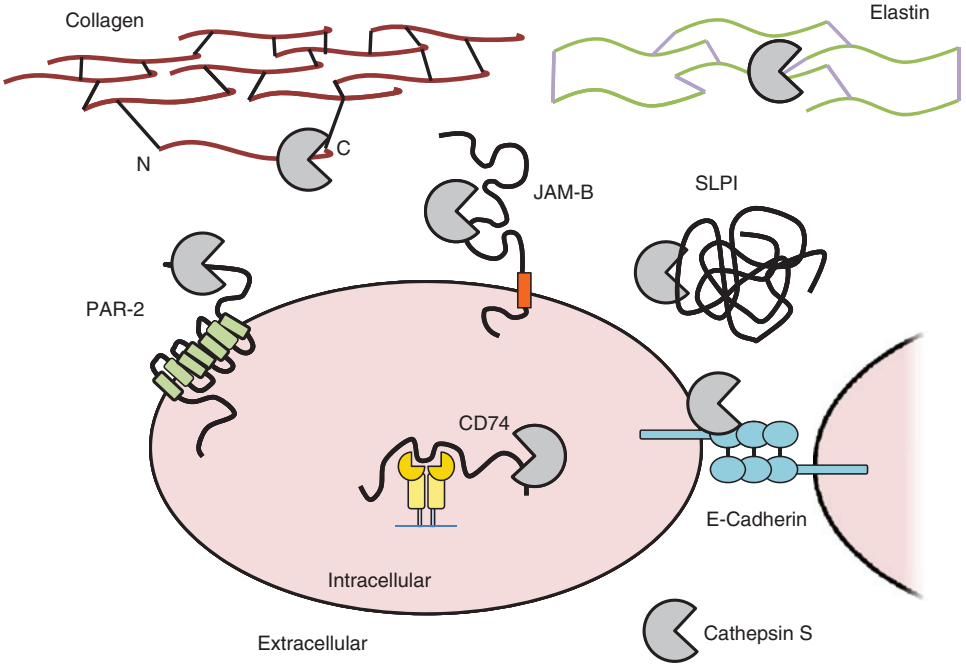

Figure 1: Substrates of cathepsin S.
Cathepsin S recognises a wide variety of substrates, both intracellularly and extracelluarly, all of which are discussed within this review. These include CD74 involved in major histocompatability complex class II (MHCII), Protease activated receptor-2 (PAR-2) involved in pain signalling, E-cadherin involved in intracellular adhesion, secretory leukoprotease inhibitor (SLPI) an endogenous neutrophil elastase inhibitor, junctional adhesion molecule-B (JAM-B) involved in cathepsin S derived blood brain barrier metastases and collagen/elastin which make up structural elements of the extracellular matrix and the basement membrane.

by the formation of a pannus which acts to irreparably destroy cartilage and erode the bone (Schurigt 2013). A major misregulation of proteases including metallo-, serine and cysteine proteases appear to be involved in joint destruction as a result of recruitment of immune cells to the site of disease (Firestein, 2005).

Using an antigen-induced arthritis murine model, it has been shown that cathepsin S is upregulated in this condition (Schurigt et al., 2005). The causative effect of the protease was further demonstrated in this model, revealing reduced progress of the disease in the absence of the protease species (Nakagawa et al., 1999). The pharmacological relevance of this expression of cathepsin S was revealed using an experimental inhibitor, CSI-75, which reduced disease scoring and disease progression (Baugh et al., 2011). In humans, similar trends of elevated cathepsin S levels are observed with plasma in patients with rheumatoid arthritis in comparison to normal healthy controls (Ruge et al., 2014). Analysis of cathepsin S plasma levels in this particular study suggests there is no link between cathepsin S expression and disease progression, but interestingly, other studies have found that the levels of the protease does have predictive value in the progression of rheumatoid arthritis (Požgan et al., 2010).

Multiple sclerosis (MS) is an inflammatory autoimmune disease whereby components of the myelin sheath protein on neurones within the brain and spine are targeted by the host immune system, inducing demyelination, resulting in subsequent neurodegeneration. The symptoms of MS can come in the form of sporadic isolated relapsing attacks or develop progressively over time leading to the onset of gradual physical, mental and psychiatric disability. A key process in demyelination is the proteolytic degradation of its constituent protein, myelin basic protein (MBP). It has been shown that cathepsin S can cleave MBP (Beck et al., 2001) and given it is an inflammatory disorder mediated by immune cells, it is hypothesised that the protease could be a key causative agent in the progression of the disorder. More recently, this initial mechanistic work has been complemented by clinical studies, where an increase in cathepsin S expression at both transcriptional and translational levels was revealed in MS patients. Relapsing-remitting MS (RR-MS) patients showed a 74% and 66% increase in cathepsin S expression vs. normal controls with regards to RNA expression and serum protein levels respectively (Haves-Zburof et al., 2011).

An important factor when examining elevated protease levels in disease is to consider the relative levels of anti-proteases. Interestingly, in a study examining these parameters in RR-MS patients, it has been shown that both cathepsin S and cystatin C levels are raised, but that the levels of the protease overwhelm the cystatin C levels, resulting in a net increase in proteolytic activity. This phenomenon is not replicated in MS patients who are currently in remission, suggesting increased cathepsin S activity to be associated with relapse of MS. Further examination carried out on peripheral blood cells of RR-MS patients suggests that cathepsin S maturation is driven by an interleukin-16 mediated signalling pathway, which may consequently augment physical symptoms of MS in patients (Martino et al., 2013).

Allergic inflammation and asthma

Asthma is a chronic inflammatory disease of the airways which causes a reduced level of reversible airflow, characterised by breathlessness, coughing and wheezing. The inhalation of allergens can stimulate the recruitment of inflammatory cells into the lung with persistent inflammation leading to the development of permanent airway remodelling and subsequent loss of function over time.

Due to its role in the antigen presentation pathway, cathepsin S may be potentially contributing to the progression of asthma pathology (Cimerman et al., 2001) which is evidenced from a number of pre-clinical models. Gene expression profiling of both BALB/C and C57BL/6J mice challenged with ovalbumin (OVA), a classic murine model for allergic lung inflammation, showed increased cathepsin S gene expression of 4.0-fold and 3.2-fold respectively (Lewis et al., 2008). A separate study examining protein levels, found that BALF cathepsin S was increased upon OVA challenge in mice (Fajardo et al., 2004). OVA challenge in *Ctss*$^{-/-}$ knockout mice did not result in pulmonary inflammation. Furthermore, treatment of wild-type mice with a reversible cathepsin S inhibitor reduced inflammation in OVA challenged mice, comparable to levels in the cathepsin S knockout model, highlighting the drugability of the protease in this disease (Deschamps et al., 2011).

Diabetes and obesity

Type 2 diabetes is characterised by the development of insulin resistance as well as a partial deficiency in insulin production. Type 2 diabetes develops later in life and progresses as a result of a complex variety of factors presented at both genetic and environmental levels.

Exploration into the role of cathepsins in development of type 2 diabetes originated from investigation of a variety of pro-inflammatory proteins, including cathepsin S, in non-obese diabetic mice. The study revealed an

increased level of cathepsin S at the mRNA level, as well as increased levels of protein as determined by immunofluorescence and activity (Li et al., 2010). In a separate study, deletion of cathepsin S in mice was shown to significantly attenuate the onset of diabetes, thus providing a level of protection (Hsing et al., 2010).

In a study examining the effect of statin treatments on atherosclerosis, rats were treated with atorvastatin over a 12-week period. A cDNA library was generated and differential gene analysis was carried out by high-throughput microarray. Cathepsin S was shown to be upregulated in diabetic rats vs. normal healthy rats. Interestingly, treatment with atorvastatin reduced cathepsin S expression in diabetic rats, further suggesting cathepsin S holds an important role in diabetes and that atorvastatin treatment can reduce its expression (Xu et al., 2013).

Cathepsin S levels have also been found to correlate with blood glucose levels *in vivo*. Cathepsin S knockout and control wild type C57BL/6J mice were fed high-fat diets over a period of up to 20 weeks. Interestingly, knockout of cathepsin S resulted in a reduced level of blood glucose in comparison to the wild type controls for both normal chow and high-fat diet mice. This effect was replicated upon treatment of wild-type mice with selective cathepsin S small molecule inhibitors. This observation highlights cathepsin S as a potential target for the normalisation of blood glucose levels in patients, delaying the progression of diabetes (Lafarge et al., 2014).

Similarly, a link between cathepsin S and obesity has also been observed where levels of the protease are increased alongside adipocyte markers in the tissues of obese patients. The addition of a cathepsin S selective inhibitor, Z-Phe-Leu-COCHO, caused a 2-fold decrease in lipid content and adipocyte marker expression in differentiated cells. The treatment of preadipocytes with recombinant cathepsin S conversely enhanced adipogenesis which taken together are indicative of a causative role for the protease in obesity (Taleb et al., 2006).

Consistent with this observation, Naour and co-workers have described a 2-fold increase in cathepsin S mRNA in adipose tissue removed from obese patients. Furthermore, cathepsin S protein was detectable in serum and the increase levels of the protease were again found in obese patients in comparison to non-obese patients. Interestingly, this study also reveals that levels of the protein in serum were reduced after weight reduction following gastric sugery (Naour et al., 2010). These findings are consistent with a more recent study showing that levels of the protease can be influenced by diet. Significantly increased levels of serum cathepsin S were observed in subjects consuming a habitual western diet as opposed to subjects consuming a healthy Nordic diet, suggesting levels may be mediated by weight loss (Jobs et al., 2014).

Cardiovascular disease

Cardiovascular disease encompasses several conditions associated with pathological processes involving the heart and vascular system. The overall maintenance of this system is driven by a protease: anti-protease balance. Although MMPs have been widely investigated (Papazafiropoulou and Tentolouris, 2009), cathepsin S has emerged as an important player in progression of pathogenesis in a range of cardiovascular diseases, such as atherosclerosis, abdominal aortic aneurysm and cardiac repair post-myocardial infarction (Chen et al., 2013a).

Atherosclerosis results from lipoproteins, such as cholesterol, accumulating at susceptible sites in the arterial vasculature. The presence of these lipoproteins encourages a pro-inflammatory immune response facilitated by recruitment of macrophages, fibroblasts and other cell types onto the arterial walls forming an atheroma. Ingestion of the lipoproteins results in generation of foam cells which form plaques and subsequently affects control of the immune cells which continue to contribute to plaque formation. Consistent plaque formation over many decades can affect the sheer flow of blood through the vasculature and lead to the formation of a clot, resulting in myocardial infarction (Libby, 2002).

Expression levels of cathepsin S are elevated in atheromas and in surrounding tissues compared to normal healthy tissues, most likely due to recruitment of protease rich immune cells (Sukhova et al., 1998). *In vivo* analysis revealed knockout of cathepsin S in mice reduced plaque formation (Sukhova et al., 2003). Furthermore, treatment of high-fat diet mice with the angiotensin II type 1 receptor (AT1) antagonist olmesartan, reduced both atheroma progression and cathepsin S protein levels *in vivo*. This is believed to be as a result of olmesartan blocking macrophage recruitment to the disease site, providing a potential molecular explanation for cathepsin S overexpression via the AT1 receptor in atherosclerotic lesions (Sasaki et al., 2010).

Abdominal aortic aneurysm (AAA) is a severe vascular disease, which remains relatively asymptomatic. Failure to diagnose the condition results in a vascular rupture, resulting in heavy internal bleeding. The onset of AAA is characterised by the destruction of integral elastin and collagen present in the vascular walls. Although the pathogenesis of the disease is typified by the presence

of a pro-inflammatory infiltrate as well as an increase in neoangiogenesis and induced apoptosis, the underlying cause of AAA remains poorly understood. Immunohistochemical analysis of AAA patient samples has revealed upregulation of cathepsin S when compared to healthy control samples (Lohoefer et al., 2012). Furthermore, in an *Apoe*$^{-/-}$ *Ctss*$^{-/-}$ double knockout mouse model of AAA, cathepsin S deficiency revealed reduced disease progression vs, *Apoe*$^{-/-}$ *Ctss*$^{+/+}$ controls (Qin et al., 2012).

Cancer

Cancer may be described as the uncontrolled proliferation of undifferentiated cells, forming a tumour. In reality, cancer is much more complicated and involves the loss of control of several hallmark features (Hanahan and Weinberg, 2011). Proteases contribute to several of these pathological pathways, such as tissue remodelling, neoangiogenesis, and cytokine production.

The cysteine cathepsins have long been implicated in the progression of cancer with the majority of the focus placed on cathepsins B, L, and S. Cathepsin S has been shown to possess an important role in angiogenesis, with cathepsin S null mice exhibiting impaired microvessel growth with respect to wound healing (Shi et al., 2003). Cathepsin S has also been shown to release other angiogenesis modulating peptides such as pro-angiogenic γ2 from anti-angiogenic peptide laminin-5 and endostatin from the NC-1 domain of type XVII collagen, suggesting a role in the modulation of microvessel growth (Wang et al., 2006; Veillard et al., 2011).

Examination of the role of cathepsin S with respect to tumour biology using genetic deletion models has revealed that knockout of the protease not only results in a reduction of angiogenesis, but also a reduction in tumour invasion and volume. These effects are complemented by increased apoptosis, probably a consequence of tumour starvation, and taken together highlights an important role for cathepsin S in tumour growth and progression (Gocheva et al., 2006). Inhibition of cathepsin S using a selective monoclonal antibody Fsn0503 showed a reduction in cell invasion in colorectal, prostate, and breast cell lines, as well as demonstrating attenuation of angiogenesis. Fsn0503 inhibition of cathepsin S *in vivo* has shown a significant decrease in tumour growth in colorectal tumour models as a single agent (Burden et al., 2009; Vázquez et al., 2014), or in combination with chemotherapy (Burden et al., 2012).

A major source of cathepsin S in tumours is from tumour associated macrophages (TAMs). In pancreatic islet tumours (RIP-Tag2), the presence of the protease appears dependent on IL-4, suggestive of association with an M2-like TAM phenotype (Gocheva et al., 2010). More recently, studies using a syngeneic model of colorectal cancer (MC38) have revealed that both tumour and tumour associated cells are both responsible for contributing the protease to the complex tumour microenvironment (Small et al., 2013). This study has been further complemented by similar findings using an invasive and metastatic breast cancer cell line model (Sevenich et al., 2014). This latter study has also revealed that cathepsin S promotes tumour brain metastasis; a consequence of cathepsin S processing of junctional adhesion molecule JAM-B in the blood brain barrier, allowing extravasation of the tumour cells into the brain. Crucially, inhibition of cathepsin S in this model significantly attenuated brain metastasis, advocating cathepsin S as a potential target to reduce formation of secondary tumours (Sevenich et al., 2014).

Although the reasons underpinning up-regulation of cathepsin S in human tumours remains unresolved, a recent analysis of 3131 tumour samples representing 26 cancer types has demonstrated a focal point of somatic copy number amplification at chromosome 1q21.2, the loci containing the cathepsin S gene. Present at the same loci is Mcl-1, a member of the BCL-2 family of anti-apoptotic proteins. The study suggests a reliance of tumours on Mcl-1, enhancing tumour cell survival. As such, the region is consistently somatically amplified, possibly collaterally amplifying cathepsin S (Beroukhim et al., 2010).

Pain

Chronic pain can be broken loosely into two categories, nociceptive and neuropathic pain. Nociceptive pain typically is characterised by injury or by disease which causes damage to tissues, such as bone and cartilage degradation in rheumatoid arthritis. Neuropathic pain is brought about by damage to the nerves themselves and can be as a result of injury, disease, or environmental factors. The boundaries of nociceptive and neuropathic pain overlap and in such instances patients may suffer from pain derived from damage to both tissues and nerves (Gangadharan and Kuner, 2013).

Upon injury, microglial cells present within the central nervous system can evoke an enhanced sensitivity and generate chronic pain via release of pro-inflammatory factors (Zhuo et al., 2011). Experimental inhibition of microglial cell activation results in a reduction of hyperalgesia and allodynia, highlighting a therapeutic rationale to dampening of these immune cells

(Tan et al., 2009). However, the feasibility of targeting microglia, risks side-effects produced by off-target inhibition of other immune cells, or indeed the nerves themselves. As such, alternative targeting strategies have been explored.

Fractalkine, a key pain-mediating chemokine, is a transmembrane protein generated by neurons and when cleaved from the neuronal membrane, binds to its receptor, CX3CR1, present on the surface membrane of microglial cells. Following peripheral nerve injury, cathepsin S has been shown to upregulated in microglial cells and secreted. It has now been shown that this extracellular cathepsin S can cleave or shed membrane-bound fractalkine into a soluble form, promoting the propagation of pain signalling. Using an irreversible cathepsin S inhibitor, morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LHVS), it has been shown that levels of hyperalgesia and allodynia in rats were significantly reduced (Clark et al., 2007). Furthermore, administration of recombinant cathepsin S protein into *Cx3cr1*$^{-/-}$ knockout mice showed no induction of allodynia as opposed to *Cx3cr1*$^{+/+}$ rats, further validating the role of cathepsin S in this pain-activating pathway (Clark et al., 2009).

A second mechanism by which nociceptive pain may be triggered is via activation of protease-activated receptor-2 (PAR2). PAR2 is a unique G-protein coupled receptor, which upon cleavage reveals a tethered ligand domain that interacts with its receptors. As PAR2 is expressed on nociceptive neurons, its activation stimulates Ca^{2+} release of neuropeptides that contribute to and cause inflammation. Cathepsin S expression has been shown to promote itch in a PAR2/4-dependent manner (Reddy et al., 2010; Elmariah et al., 2014), and similarly activation of transient receptor potential vanilloid 4 (TRPV4) through PAR2 (Zhao et al., 2014).

Neuropathic pain is a major symptom in colitis, an inflammatory colon disease. Cathepsins S, B, and L have been shown to be upregulated in an *Il10*$^{-/-}$ murine model of chronic colitis. Not only is cathepsin S overexpressed in the disease state, but it is also secreted. In C57BL/6 mice, addition of recombinant cathepsin S into the colonic lumen produced higher levels of nociceptive pain when compared against vehicle and basal controls. Pre-treatment of recombinant cathepsin S pre-incubated with LHVS, negated the nociceptive pain observed in the mice, indicating a requirement for cathepsin S. Interestingly, hyperalgesia was shown to be fully attenuated in *Par2*$^{-/-}$ mice, with cathepsin S levels shown to be significantly reduced in *Par2*$^{-/-}$ mice vs. *Par2*$^{+/+}$ mice (Cattaruzza et al., 2011).

Cystic fibrosis

Cystic fibrosis (CF) is a chronic inflammatory disease, characterised by the destruction of lung elasticity due to an imbalance in protease: anti-protease expression. Cathepsin S is found expressed in both the upper and lower airways of CF patients where it has been shown to promote the disease through a variety of mechanisms (Hentschel et al., 2014). For example, the protease has been shown to cleave secretory leukoprotease inhibitor (SLPI), an endogenous inhibitor of neutrophil elastase which plays a pivotal role in elastin degradation in the CF lung (Taggart et al., 2001). Cathepsin S activity is not correlated with *Pseudomonas aeruginosa* infection status (Naudin et al., 2011), but it has been implicated in the degradation of defensins, protective proteins that exhibit an antimicrobial effect against pseudomonas in CF patients, and surfactant protein A, a protein which holds an innate protective role, contributing to the progression of CF symptoms (Taggart et al., 2003; Lecaille et al., 2013). Proteolytic processing of amiloride-sensitive epithelial sodium channel by cathepsin S may also aggravate the physiological symptoms presented in CF patients (Haerteis et al., 2012).

Interestingly, a recent study by Weldon et al (2014), has revealed a new mechanism through which cathepsin S expression becomes elevated in CF BAL fluid of non-*Pseudomonas aeruginosa* infected patients. Epithelial cells were found to drive expression of the protease in an IRF-1 dependent manner that was a result of the loss of miR-31 which negatively regulates the expression of this transcription factor (Weldon et al., 2014).

The therapeutic utility of cathepsin S

Interest in Cathepsin S as a target has led to the development of a wide range of low molecular weight, small molecule compounds (Jedinak and Maliar, 2004). These compounds have been designed to engage the active site of the target protease and bind to catalytic cysteine residue via the employment of a warhead that is typically electrophilic in nature (Chang et al., 2007). The types of warheads that have been utilised are either irreversible (epoxide, Michael acceptor, α-haloketone) or reversible (cyano, ketoamide) in design (Lee-Dutra et al., 2011).

One fundamental issue with targeting the cathepsins is that the family shares high homology. Therefore, inhibitors require significant optimisation in order to identify a compound that displays sufficient selectivity. The determination of cathepsin S: inhibitor complex structures by

X-ray crystallography, has allowed for detailed analysis of the substrate binding cleft of the protease active site (McGrath et al., 1998; Pauly et al., 2003). From this pioneering work, it is now known that cathepsin inhibitor selectivity can be developed via the optimal occupation of the S2 and S3 sub-sites, as well as overcoming gatekeeper residue interactions into the pockets (Turk et al., 2012b).

A second issue with cathepsin inhibitors is lysomotrophism; the accumulation of weakly basic and lipophilic compounds in the lysosomal compartments of the cell (MacIntyre and Cutler, 1988). The accumulation of these compounds into the endo-lysosomal pathway causes increased off-target binding of the inhibitor, reducing efficacy. As an additional consequence, the sequestering of weakly basic compounds in the acidic lysosomal environment may increase the pH and may lead to swelling of the lysosomal compartment, thereby disrupting the functional roles of the organelle.

The effect of lysomotrophism on inhibitor efficacy was a major barrier in the development of early cathepsin K inhibitors (Black and Percival, 2006). Cathepsin K holds a physiological role in bone resorption, and pathologically has been implicated in osteoarthritis (Logar et al., 2007). As such, cathepsin K has become an attractive target for the development of new treatments. Early cathepsin K inhibitors exhibited problems with lysomotrophism and compounds such as CRA-013783, which showed high selectivity towards cathepsin K were found to be poorly efficacious *in vivo* (Falgueyret et al., 2005). However, more recently, the cathepsin K inhibitor Odanacatib has overcome these issues and is currently undergoing evaluation in phase 3 clinical trials as a treatment for osteoporosis (Gauthier et al., 2008; Zerbini and McClung, 2013).

The progress in overcoming the problems of lysomotrophism in cathepsin K compounds has opened up the potential for targeting other cathepsin proteases such as cathepsin S. Cathepsin S inhibitor LY3000328 has recently completed phase I clinical trial evaluating safety, tolerability, and pharmacodynamics in human subjects. It demonstrated a transient reduction of cathepsin activity in the plasma, interestingly followed by a rise in overall cathepsin S protein levels and subsequent clearance of LY3000328 (Payne et al., 2014). Several cathepsin S inhibitors are currently in clinical trials for the treatment of rheumatoid arthritis, psoriasis and neuropathic pain (Table 1). The emerging role of cathepsin S in a plethora of inflammatory diseases and tumourigenesis is likely to result in further cathepsin S compounds being clinically evaluated in the near future.

Cathepsin S as a diagnostic/prognostic marker

Not only does cathepsin S hold promise as a therapeutic target but as a result of its differential expression in disease it may also hold potential as a biomarker, predictive of disease diagnosis, and/or prognosis. Cathepsin S expression may be used to stratify patients towards treatment options and as such increase the effectiveness of current and new precision medicine therapeutic strategies.

Cathepsin S has been shown to be differentially expressed in a variety of cancers such as hepatocellular, prostate and pancreatic carcinomas, which is in direct contrast to normal distribution of cathepsin S under healthy conditions (Fernández et al., 2001; Xu et al., 2009). Furthermore, Flannery et al., evaluated the prognostic/diagnostic value of cathepsin S levels in astrocytomas, where the expression of cathepsin S in 146 brain neoplasms was evaluated by ELISA and immunohistochemistry. An increased level of cathepsin S was observed in grade IV astrocytomas, significantly higher than grade I, II and III tumours. Survival analysis revealed a significant reduction in survival for patients with higher cathepsin S immunohistochemical scoring and multivariable

Table 1: Cathepsin inhibitors currently undergoing clinical trials.

Drug	Cathepsin	Company	Stage	Disease	References
Odanacatib	K	Merck	Phase III	Osteporosis	http://www.mercknewsroom.com/press-release/research-development-news/new-data-odanacatib-mercks-phase-iii- investigational-cat-k-i
RWJ-445380	S	Johnson & Johnson	Phase II	Rheumatoid arthritis	http://clinicaltrials.gov/ct2/show/study/NCT00425321
VBY-036	S	Virobay Inc.	Phase I	Neuropathic pain	http://clinicaltrials.gov/ct2/show/NCT01892891
VBY-891	S	Virobay Inc., LEO Pharma	Phase I	Psoriasis	http://clinicaltrials.gov/ct2/show/NCT01947738
CRA-028129	S	Celera	Phase I	Psoriasis	https://www.celera.com/celera/pr_1127168705

analysis showed cathepsin S to be a significant variable with respect to prognostic value (Flannery et al., 2006).

Gormley et al., have evaluated the prognostic/diagnostic value of cathepsin S in colorectal cancer. A large grouping of 560 patient samples made up of three cohorts was analysed by tissue micro-array. Immunohistochemical scoring of cathepsin S in these samples revealed expression in more than 95% of the samples, with a 1.3-fold increase in cathepsin S in tumours vs. normal tissues. Survival analysis also revealed a correlation between survival and cathepsin S expression, with high cathepsin S expression being indicative of poorer prognosis (Gormley et al., 2011).

The ability to detect cathepsin S levels quantitatively in patient serum has recently become an area of intensive research. In diabetes, cathepsin S serum levels have been shown to be increased when compared to normal healthy patient serum (Chen et al., 2013b; Jobs et al., 2013). Interestingly a correlation between diabetes and endogenous cathepsin inhibitor cystatin C was also observed (Liu et al., 2006). Analysis of plasma samples from atherosclerosis patients, revealed higher cathepsin S expression in comparison to normal control serum (Lv et al., 2012). Furthermore, patients with myocardial infarction, both acute and prior myocardial infarction, or unstable angina, exhibited higher cathepsin S serum levels. Increased serum cathepsin S levels have also been linked with adiposity, thus compounding the evidence linking obesity and atherosclerosis (Taleb et al., 2005). Finally, cathepsin S serum levels in abdominal aortic aneurysm patients has been found to correlate strongly with high-sensitivity C-reactive protein (hs-CRP), a marker of AAA disease progression, suggesting combined cathepsin S and hs-CRP analysis may provide a more sensitive predictive marker of AAA in a clinical setting (Qin et al., 2013).

Interestingly, new research has indicated that lacrimal fluid may also be used to measure cathepsin S as a biomarker for Sjögren's syndrome, an autoimmune disease that affects lacrimal and salivary glands resulting in a drying of the eyes and mouth. The study showed cathepsin S levels to be 4.1-fold higher in patients with Sjögren's syndrome vs. patients with other autoimmune diseases and 41.1-fold higher than normal healthy patients (Hamm-Alvarez et al., 2014).

Accurately determining cathepsin S activity

Presently, cathepsin S expression may be observed via biopsy of tumours or interrogation of serum samples. Traditionally, observation of biomarkers has been carried out using protein quantification techniques such as immunohistochemistry (Capelozzi, 2009). More recently, Cox et al., have described a robust and powerful method measuring cathepsin S levels in the serum by ELISA and LC-MS/MS, which has been utilised as method to detect levels of the mature active form of cathepsin S in human serum (Cox et al., 2012).

These quantitative strategies however fall short for two reasons; failure to define mature active protease from inactive and/or pro-form and a lack of tools to sensitively and accurately report the relevant active protease levels. Cathepsins may exist within a variety of forms on route to maturation, where at any time they may be present in an inactive pro-form. Furthermore, once activated cathepsins may be secreted from the endo-lysosomal system and become inactivated by endogenous inhibitors or by oxidation of the thiol group present in the active site (Hervé-Grépinet et al., 2008). Current methods evaluating cathepsin levels include substrate assays measuring protease activity and immunological assays such as ELISA to measure protein levels. Unfortunately, due to the wide substrate promiscuity of the cathepsins and an inability to discriminate between active and inactive cathepsin protein, substrate-based activity assays and ELISAs cannot provide selective and sensitive readouts of cathepsin activity in disease.

The emergence of protease activity profiling is a relatively new concept in quantitative biology. Within disease, the sheer abundance of proteins present within target tissues alongside the complexity of protein post-translational modifications tends to skew most qualitative and quantitative assays based on measuring functionally active proteases. Profiling based on enzymatic activity provides clearer visualisation of cellular activities controlled by proteolysis.

Much focus has been placed on the development of substrates and probes that provide selective and sensitive measurement of protease activity within disease (Mahmood et al., 1999; Bremer et al., 2002; Berger et al., 2004). The use of substrates to quantify proteolytic turnover has been utilised for decades, isolating, identifying, and quantifying products for analysis. The development of substrate-based probes takes advantage of a signal element which becomes activated upon cleavage by a target protease. Substrate-based probes are made up of a protease recognition sequence, which helps infers selectivity towards a target protease and a signal moiety, which allows quantification via non-invasive procedures. One example of a substrate-based probe is ProSense 680, a broad-spectrum polymeric substrate containing a near

infrared fluorophore (Grimm et al., 2005). Prosense680 is a quenched substrate-based probe, requiring cleavage and separation of the quenching unit from the fluorophore before transmission of signal. It has been applied to a variety of inflammatory processes including asthma, angiogenesis and the growth of intestinal polyps, where it has used for visualise the contribution of cathepsin B in these diseases (Cortez-Retamozo et al., 2008; Gounaris et al., 2008).

More recently, a new generation of 'reverse design' substrate-based probes have been developed. Reverse design substrate-based probes take advantage of extensive knowledge of selective inhibitors originally developed for and pharmacologically optimised for therapeutic purposes. In place of an active warhead moiety targeting the protease active site is a peptide bond linking the inhibitor to a fluorogenic signal element (Watzke et al., 2008). One such compound, AW-091, has already demonstrated a high selectivity towards cathepsin S and allows for sensitive visualisation of cathepsin S rich areas *in vivo* (Caglič et al., 2011). This reverse design approach has also been successfully applied by Hu et al., whereby highly selective cathepsin S inhibitors were adapted into lipidated and non-lipidated substrate probes and utilised *in vivo* to target grafted murine mammary adenocarcinoma cells (4T1). The study demonstrates strong visualisation of the tumour site *in vivo* with very little noise generated as a result of substrate deposition in off-target tissues. Furthermore, signal was rapidly produced within 30 min, which increased steadily over the following 6 h before diminishing 24 h post-injection (Hu et al., 2014).

Activity based probes (ABPs) can also be utilised to provide a novel method for analysing protease activity within disease by selectively and covalently binding to active proteases and allowing visualisation within the disease site. ABPs are comprised of three distinct parts; a warhead domain that binds covalently to the protease of interest, a spacer containing a sequence recognition domain conferring specificity and a tagged signal element that is used to quantify the level of protease present. With respect to the cysteine cathepsin family, selective ABPs against cathepsins B, L, and S have been designed. These ABPs carry an irreversible warhead such as acyloxymethyl ketone (AOMK) and can be cell permeable. The AOMK ABPs can selectively bind to and label individual cathepsins in cells and lysates and can be visualised by fluorescent SDS-PAGE (Blum et al., 2005). Visualisation of ABPs in an *in vivo* environment has also been demonstrated using ABP-treated xenograft models, with detectable protease activity diminished following treatment with a cathepsin inhibitor (Blum et al., 2007). Furthermore, a non-peptidic ABP, BVM109, has demonstrated remarkable ability with respect to visualisation of cathepsins X, B, L, and S *in vivo*. BVM109 provides an improvement on previously described peptidic quenched ABP, GB137, revealing a more than 20-fold higher contrast imaging of tumour sites *in vivo* over a longer time period (Verdoes et al., 2013). More recently, development of a quenched cathepsin S-selective ABP BVM157, has demonstrated high selectivity towards cathepsin S over X, B and L. BVM157 provided excellent *in vivo* visualisation of tumours in 4T1-tumour bearing mouse with *ex vivo* analysis confirming selectivity of the probe towards cathepsin S when compared to BVM109 (Oresic Bender et al., 2015). This demonstrates a novel and sensitive way in which protease activity may be monitored using ABPs for potential diagnostic and prognostic value and also provide a non-invasive sensitive readout of treatment effectiveness (Figure 2). The use of positron emission tomography (PET) radio-nucleotide labelled AOMK probes has been successfully utilised in nude mice, allowing visualisation of tumours in a non-invasive manner. Furthermore, sensitivity of PET visualisation has been demonstrated by addition of cysteine cathepsin inhibitor, abrogating probe sensitivity (Ren et al., 2011).

A comparative analysis of ProSense substrate-based probes vs. AOMK ABPs shows the differences with regards to *in vivo* visualisation using non-invasive techniques. The results demonstrated a slower and subsequently lower level of signal for the ProSense substrate-based probes in comparison with the activity-based probes (Blum et al., 2009). One major drawback of substrate-based probes is protease turnover of substrate is not on a one-to-one basis, meaning that a single protease molecule may potentially turn over hundreds of substrate probes and accurate quantitative readouts may not be obtained. Conversely, ABPs suffer from being irreversibly covalent and as such, once bound, cannot be released, therefore potentiating disruptions to pathways (Edgington et al., 2011).

Nanocarrier systems are an interesting approach to providing *in vivo* localisation of drugs and probes into tumours, exploiting the ability of macromolecular molecules to passively accumulate in tumour microenvironments through enhanced permeability and retention (EPR) effects (Fay and Scott, 2011). Nanocarriers containing surface conjugated probes targeting proteases have provided a unique mechanism by which tumours may be visualised. One such study demonstrates the use of a cathepsin B inhibitor, LNC-NS-629, which has been tethered onto a liposomal nanocarrier primarily for the delivery of adjuvant therapy to tumour sites. Using a murine pancreatic tumour model, effective internalisation of LNC-NS629 into endosomal compartments has been demonstrated.

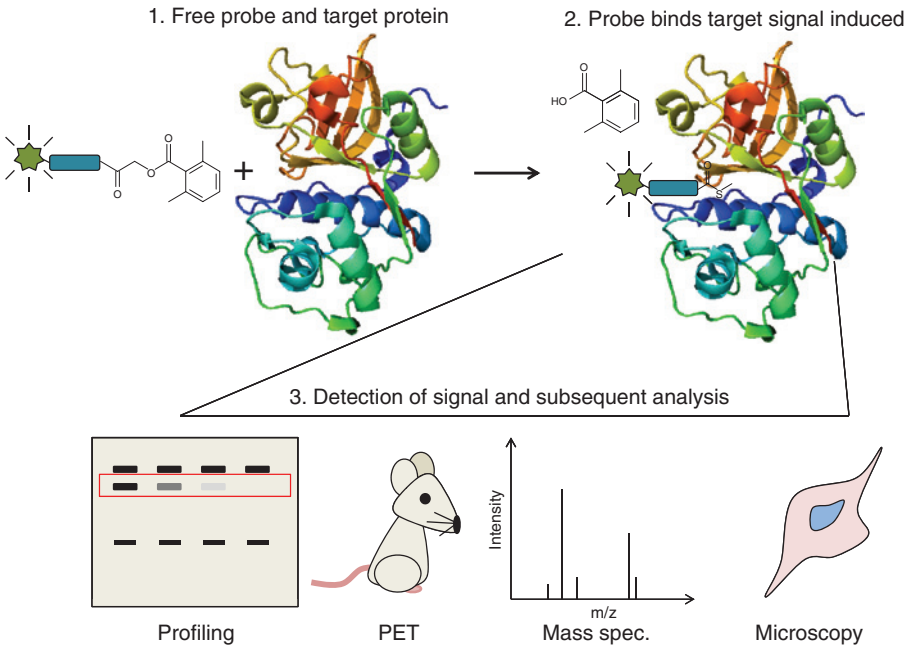

Figure 2: Versatility of substrate-based probes and activity-based probes.
Profiling of proteases using substrate- and activity-based probes may take advantage of several existing visualisation techniques allowing qualitative and quantitative analysis. These include Western blotting, positron emission tomography (PET), mass spectrometery, and microscopic analysis.

Furthermore, this methodology allows for the visualisation of tumour sites, via encapsulation of Magnevist, a magnetic resonance imaging contrast agent. Use of Magnevist enhanced visualisation of the tumour site *in vivo* and continued to provide signal 24 h after administration, demonstrating the potential value of liposomal nanocarriers containing targeting entities as diagnostic tools (Mikhaylov et al., 2014).

Perspectives

In the last 20 years, there has been a widening understanding of cathepsin S and its role in disease. Early cathepsin S inhibitors showed remarkable promise in autoimmune disease, however due to a lack of clinical success, interest for these inhibitors as novel therapeutics waned.

In recent years, emerging evidence for a role in other pathological conditions such as pain, colitis and cancer has reinvigorated interest in cathepsin S. These new links between cathepsin S and disease have generated much interest in cathepsin S as a therapeutic target and a number of selective inhibitors from various pharmaceutical companies have now entered clinical trials.

Current methods being used to investigate the mechanistic role of cathepsin S in disease are proving inadequate, with many pathways remaining poorly defined. The recent publication of several studies showing that differential cathepsin S expression holds a diagnostic and/or prognostic value in these diseases further highlight the need for new exploratory tools to elucidate these mechanistic roles.

Activity profiling tools provide an exciting and novel area for exploiting predictive information from patients by providing a readout of protease levels in disease. These techniques overcome difficulties such as lack of protease selectivity and lack of definition between pro-form and mature forms of protease experienced under substrate activity assays and immunoassays.

In conclusion, the increasing interest in cathepsin S as a therapeutic target and as a potential biomarker with diagnostic and/or prognostic value can only accelerate the development of new clinical inhibitory and detection technologies for application in a variety of pathological conditions.

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