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Research paper

Biochemical properties and regulation of cathepsin K activity

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Abstract

Cysteine cathepsins (11 in humans) are mostly located in the acidic compartments of cells. They have been known for decades to be involved in intracellular protein degradation as housekeeping proteases. However, the discovery of new cathepsins, including cathepsins K, V and F, has provided strong evidence that they also participate in specific biological events. This review focuses on the current knowledge of cathepsin K, the major bone cysteine protease, which is a drug target of clinical interest. Nevertheless, we will not discuss recent developments in cathepsin K inhibitor design since they have been extensively detailed elsewhere. We will cover features of cathepsin K structure, cellular and tissue distribution, substrate specificity, and regulation (pH, propeptide, glycosaminoglycans, oxidants), and its putative roles in physiological or pathophysiological processes. Finally, we will review the kinetic data of its inhibition by natural endogenous inhibitors (stefin B, cystatin C, H- and L-kininogens).

Keywords: Cystatin; Cysteine cathepsin; Cysteine protease; Kininogen; Stefin; Substrate specificity; Osteoporosis

1. Introduction

The complete sequence of the human genome, published in 2003, encoded at total of 11 cysteine cathepsins (B, H, L, S, C, K, O, F, V, X and W). These, which are also known as lysosomal proteases, belong to the papain-like protease family (clan CA, family C1) (MEROPS: the peptidase database: http://merops.sanger.ac.uk; [1]). Cathepsin K has been the focus of considerable attention for the last ten years, since it is the major cysteine cathepsin in osteoclasts and is involved in the digestion of extracellular matrix during bone remodeling. Thus, pharmaceutical companies have become keenly interested in cathepsin K, as the selective inhibition of cathepsin K could well be the key to an effective treatment of diseases with excessive bone resorption such as osteoporosis. This review will not discuss the developments of synthetic cathepsin K inhibitors since this topic has been extensively detailed elsewhere. Interested readers should refer to the following reviews [2–8]. Several cathepsin K inhibitors have been developed by major pharmaceutical companies and are currently in clinical trials. Hence, further studies to improve the potency and selectivity of these inhibitors appear to be crucial. We will focus on current knowledge of the biochemical properties of cathepsin K and its putative involvement in several physiological and pathological processes. We will also discuss the regulation of cathepsin K activity by pH, propeptide, glycosaminoglycans, oxidants and the actions of natural endogenous inhibitors.

2. Cathepsin K: sequence, folding, catalytic mechanism, substrate specificity and synthesis

2.1. Nomenclature

Willstatter and Bamann first used the term cathepsin to designate one of the intracellular proteinases [9]. Since then, the

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literature on cathepsins has grown rapidly and become quite confused. More than 20 enzymes have been labeled cathepsins, regardless of their classification. All known lysosomal cysteine proteases are cathepsins, but not all cathepsins are lysosomal or cysteine proteases (CPs). For instance, cathepsins A and G are serine proteases, while cathepsins D and E are aspartyl proteases. The remaining letters of the alphabet refer to cysteine proteases. However, particular enzymes have been given multiple names. For instance, the cathepsin K gene was identified by several different groups and originally cloned from rabbit osteoclasts. It was formerly given the name cathepsin OC-2 [10]. Human cathepsin K has also been described as cathepsin O in human monocyte-derived macrophages [11], but as cathepsin K in human tissues [12,13], as cathepsin X in osteoclasts [14] and as cathepsin O2 in osteoclastomas and ovaries [15]. Cathepsin K was also cloned from chicken tissues and named JTAP-1 [16].

2.2. Genomic organization and regulation

Human cathepsin K is encoded by approximately 12.1 kb of genomic DNA and was mapped to chromosome 1q21 (like cathepsin S) by fluorescence *in situ* hybridization (FISH) [17,18]. Analysis of the genome DNA sequence indicates eight exons and seven introns, with an organization similar to that of cathepsins L and S [19]. Like cathepsin S, human cathepsin K is encoded by a single-copy gene, unlike cathepsin L, which is encoded by multiple species of mRNA and pseudogenes [18,19]. No TATA/CAAT box has been found at the 5′ end of the transcriptional initiation start, but two consensus Sp1 binding sites and a rich G+C region (42.5%) were identified in the promoter region as potential regulatory elements. Initiation of transcription may be enhanced by several putative transcription regulatory elements: AP1, AP3, H-APF-1, PU.1, ETS-1, PEA3, Mitf, TFE3 [18,20–23]. A NF-κB binding site has been found in the cathepsin K promoter [24]. Like cathepsin K, ETS-1 is present in the adult mouse lung and bone marrow [25], while PEA3/ETS-1 and AP1 clusters have been found to be essential for transcriptional activation upon induction of Ras in ovarian tumor-derived OVCAR 3 cells [26]. PU.1 is involved in cathepsin K gene regulation in macrophages [27], and the transcription factors PU.1 and MITF both regulate specific genes in response to colony-stimulating factor 1 (CSF-1) and receptor activator of the NF-κB ligand (RANKL) signaling during osteoclast differentiation. Furthermore, interferon (IFN)-γ and interleukins (IL)-6 and -13 stimulate the synthesis of cathepsin K in macrophages and smooth muscle cells via the H-APF-1 sequence, as well as cathepsins B, H, L and S. In contrast, transforming growth factor (TGF)-β1 and IL-10 block the activity of cathepsin K in most cells [28–34]. Recent results indicate that IL-1α, together with RANKL or M-CSF+RANKL, stimulate cathepsin K gene expression in osteoclasts via the NF-κB pathway [35,36]. Pro-inflammatory cytokines, including TNF-α, have an apparent positive effect on cathepsin K production by rheumatoid synoviocytes, but not by cultured human osteoblast-like cells [37–39].

The cDNA of human cathepsin K has been cloned in several heterologous systems and extensively studied [11–14,40,41]. The systems used to generate active cysteine cathepsins have been reviewed [42].

2.3. Folding

The crystal structures of mammalian cysteine cathepsins, except cathepsins O and W, have been published and are available from the PDB (see for review [43]). Lysosomal cysteine cathepsins have papain-like sequences and folds [6,44]. Mature cathepsin K (EC 3.4.22.38; Uniprot accession: P43235; MEROPS: C01.036) is a monomeric protein with an apparent molecular mass of about 24 kDa. High-resolution 3D structures of both the cathepsin K zymogen and its mature form are shown in Table 1. The organization of the 329-residue prepropeptide is typical of the members of the C1 family, with a 15-amino acid signal sequence, a 99-residue propeptide and the overall organization of the catalytic site, which consists of two domains (left and right in accordance with the orientation used in the standard view) folded together to give a ‘‘V’’-shaped active site cleft configuration. The central helix is the most prominent feature of the left domain, whereas the right domain is mostly dominated by β-barrel motifs (5–6 strands). The active site lies at the interface between the two domains. One of two putative N-linked glycosylation sites (N-X-S/T) is located in the propeptide and the other is in the mature protease. Both are conserved among cathepsin K proteins from all species sequenced to date. Alignment of the 11 human cysteine cathepsins protein sequences reveals a clustering into three major subfamilies: cathepsin B-like, cathepsin L-like, and cathepsin F-like [45]. While mature cathepsin K is not very similar to either cathepsin B (24% identity) or cathepsin F (40% identity), it has about 60% amino acid sequence identity with cathepsins L, S and V (Table 2).

While many cathepsins are negatively charged, cathepsin K has a high density of positively charged basic residues (Lys and Arg residues) located opposite the active cleft (Fig. 1). These positive charges allow for electrostatic interactions with negatively charged glycosaminoglycans (chondroitin 4-sulfate; C4-S), which contribute to the formation of a collagenolytically active complex (see further sections). Although their overall topographical structures are similar, each cysteine cathepsin has unique features that give each enzyme its specificity.

2.4. Catalytic mechanism

The catalytic triad of cathepsin K (Cys^{25}, His^{159}, Asn^{175}, papain numbering) is classically housed in a cleft separating the two domains, with Cys^{25} located in a long, conserved, N-terminal α-helix of the left domain, whereas His^{159} is in the other domain. Cys^{25} and His^{159} are believed to exist as the thiolate–imidazolium ion pair which is stabilized by Asn^{175} via a hydrogen bond with His^{159} [46]. The cysteine sulfhydryl group is partly responsible for the low pK_a

Table 1
Available crystallographic structures of cathepsin K

	PDB code	Resolution (Å)	Species	References
Procathepsin K	7PCK	3.2	Human	[200]
	1BY8	2.6	Porcine	[199]
Cathepsin K				
+E-64	1ATK	2.2	Human	[257]
+3-diacylaminomethyl ketone	1AU0	2.6	Human	[2]
+propanone	1AU2	2.6	Human	[2]
+pyrrolidinone	1AU3	2.5	Human	[2]
	1AU4	2.3	Human	[2]
+biscarbohydrazide	1AYU	2.2	Human	[258]
+thiazolhydrazide	1AYV	2.3	Human	[258]
+benzyloxybenzoylcarbohydrazide	1AYW	2.4	Human	[258]
+peptidomimetic inhibitor	1BGO	2.3	Human	[259]
+vinyl sulfone	1MEM	1.8	Human	[260]
+azepanone	1NL6	2.8	Human	[261]
+azepanone	1NLJ	2.4	Human	[261]
+aldehyde	1Q6K	2.1	Human	[262]
+aldehyde	1SNK	2.4	Human	[263]
+ketoamide	1TU6	1.75	Human	[264]
+purine nitrile	1U9V–X	2.2–2.1–2.3	Human	[265]
+cyanopyrrolidine	1YK7	2.5	Human	[8]
	1YK8	2.6	Human	[266]
+constrained ketoamide	1YT7	2.3	Human	[267]
+myocrisin	2AT0	2.0	Human	Weidauer et al. (to be published)
+semicarbazone	2AUX	2.4	Human	[268]
	2AUZ	2.3	Human	[268]
+pyrrolidine ketoamide	2BDL	2.0	Human	[269]
+peptidomimetic inhibitor	2FDZ	2.0	Human	[270]
+nitrile	2F7D	1.9	Rabbit	Somoza (to be published)

(~3.7) [47,48]. Briefly, the thiolate anion attacks the carbonyl carbon of the substrate bond to be cleaved to form a tetrahedral intermediate. This intermediate is first stabilized by the oxyanion hole and then transformed into an acyl enzyme with the release of the protonated leaving amine [49]. A nucleophilic attack by a water molecule results in the formation of second tetrahedral intermediate. This finally splits to generate the free enzyme and the second portion (R-COOH) of the substrate (for further details, see reviews [50,51]).

2.5. Substrate specificity

Most C1 cysteine cathepsins are endopeptidases (L, S, K, V, F), while cathepsin X is a carboxypeptidase and cathepsins B, C and H have both endopeptidase- and exopeptidase activities. According to Schechter and Berger, the substrate-binding region of cysteine cathepsins is defined as an arrangement of binding subsites (S–S′) for peptide substrate amino acids (P–P′) on both sides (N- and C-) of the scissile bond, encompassing the stretch of seven sites from S4 to S3′ of papain [52]. Since the crystal structure of numerous substrate analogue inhibitors are available, the definition has been revised and redefined, limiting the binding of substrate residues to subsites S2–S1′, in which both main-chain and side-chain atoms are involved [6,53]. However recent studies have shown the importance of cathepsin K site S3 for determining substrate specificity [54,55]. Whereas the S2 binding site is a true deep pocket, the other sites provide a binding surface. Furthermore, while S2 and S1′ sites are the major determinants of specificity, S1 is important for the affinity and efficient catalysis of substrates. The positioning of the P3 residue in site S3 is, as in subsite S2′, mediated only by side chain contacts over a relative wide area. Cathepsins K, L, S and V have partly overlapping specificities, making it difficult to discriminate between them *in vivo* [56]. Cathepsin K attacks sites having aliphatic amino acids (Leu, Ile, Val), unlike cathepsins L and V (which both rather accept hydrophobic residues with preference for Phe), and also accommodates Pro in the S2 subsite [48,54,55,57]. Cathepsin K is unusual among cysteine cathepsins in that it can cleave substrates with Pro in the P2 position, although it has been reported that congopain, a cysteine protease from *Trypanosoma congolense*, with an amino acid sequence (65% of homology) and biochemical properties similar to cathepsin K, also does so [58]. We therefore developed a specific peptide substrate Abz-His-Pro-Gly-Gly-Pro-Gln-EDDnp for cathepsin K ($k_{cat}/K_m = 426{,}000\ M^{-1}\ s^{-1}$; scissible bond: Gly–Gly) that is resistant to proteolysis by both cathepsins B, L S, F, H and V, and the serine proteases (cathepsin G, chymotrypsin and leukocyte elastase) [58]. Furthermore, fibroblasts from cathepsin K deficient mice do not cleave this substrate [58]. Recently, Weissleder and colleagues developed a selective near infra-red probe based on this sequence for *in vivo* cathepsin K imaging [59]. Another feature of cathepsin K is its preference for Gly at the P3 position. Aibe

Table 2
Human cysteine cathepsins organization and amino acids sequence identity with cathepsin K

Proteases	Prodomain					Mature domain				Prepro-enzyme			
AC number (Swiss-Prot)	Signal peptide (AA)	Length (AA)	Identity (%)	Size (Da)	p*I*	Length (AA)	Identity (%)	Size (Da)	p*I*	Length (AA)	Identity (%)	Size (Da)	Chromosomal position
Cathepsin K P43235	15	99	100	11833	6.3	215	100	23495	8.9	329	100	36966	1q21
Cathepsin L P07711	17	96	34	11724	9.0	220	60	24170	4.7	333	50	37546	9q21–22
Cathepsin V (L2) O60911	17	96	37	11672	9.5	221	59	23999	8.6	334	50	37329	9q22.2
Cathepsin S P25774	16	98	52	11830	9.3	217	58	23993	7.6	331	54	37479	1q21
Cathepsin O P43234	23	84	7	9894	9.8	214	35	23460	5.8	321	24	35957	4q31–32
Cathepsin F Q9UBX1	19	251	5	27846	9.4	214	40	23593	5.8	484	30	53365	11q13.1–3
Cathepsin X (Z) Q9UBR2	23	38	15	4338	9.6	242	22	27149	5.5	303	18	33868	20q13
Cathepsin B P07858	17	62	9	7245	9.6	260	24	28663	5.2	339	20	37807	8p22
Cathepsin C P53634	24	206	3	23534	8.7	233	30	26032	5.6	463	19	51841	11q14.1–3
Cathepsin H P09668	22	75	26	9104	9.8	220	48	24187	5.7	335	38	37403	15q24–25
Cathepsin W P56202	21	106	7	12060	4.8	249	29	28024	8.9	376	25	42099	11q13.1–3

and coworkers also suggested that Z-Gly-Pro-Arg-MCA could be a suitable substrate for cathepsin K ($k_{cat}/K_m = 117\,600\ M^{-1}\ s^{-1}$) [60]. However, it is also hydrolyzed by cathepsin B with a lower specificity constant ($k_{cat}/K_m = 9\,490\ M^{-1}\ s^{-1}$) [61]. These data can be useful information for designing and developing potent, selective peptide-based substrates and inhibitors. Details of the substrate specificities of cathepsin K are given in Table 3.

2.6. Zymogen activation

Cathepsin K is synthesized as an inactive glycosylated preproenzyme (37 kDa) in the rough endoplasmic reticulum. Procathepsin K is then transported to the lysosomal compartment via the mannose-6 phosphate (M-6P) receptor pathway [62]. However, several reports have described an additional, M-6P-independent, transport mechanism for lysosomal proteases in certain cell types that involves changes in the receptors or different CP glycosylation patterns [63–65]. The propeptide must be removed before enzyme activation. Activation can be catalyzed by other cathepsins, such as cathepsin D [66] or other CP-related enzymes [67] or by autocatalysis, and depends on a drop in pH and/or the presence of glycosaminoglycans, especially under physiological conditions [53,68]. A recent reports has shown that the inositol 1,4,5-triphosphate (IP3) transduction cascade (involving the cAMP-dependent PKA signaling pathway) can accelerate the processing and maturation of cathepsin K zymogen in mouse osteoclasts [69]. Since cathepsin K is a soluble protein lacking a transmembrane domain, this lysosomal cysteine cathepsin may be secreted from certain cells. Many cells bear M-6P receptors that may be involved in the binding of the glycosylated proform rather than the mature form of cathepsin K. However, the mature form has a putative glycosylation site. Other receptors may include the lipoprotein receptor-related proteins (LRPs) like gp330/megalin, especially efficient in secretion-recapture pathway in kidney [70,71]. Cell heparan sulfate may also anchor cathepsin K to the membrane and therefore increase its activity, even at neutral pH, as reported for cathepsin B [72].

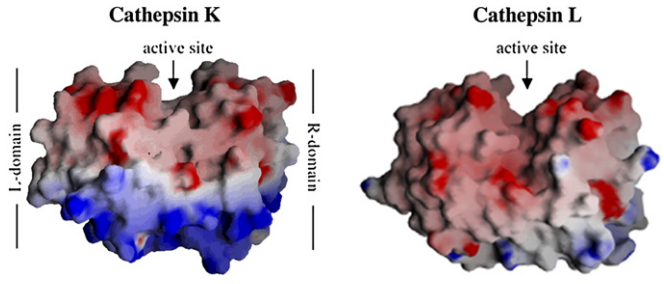

Fig. 1. Surface potential of cathepsins K and L. The electrostatic surface potential was generated with GRASP [256]. Regions of negative potential are colored red and areas of positive potential are in blue.

2.7. Tissues and cellular distribution

Cysteine cathepsins are not strictly lysosomal, the proteases are transported between phagosomes, endosomes and lysosomes, and individual enzymes may accumulate in certain organelles under specific physiological circumstances. Cysteine cathepsins are also released into the cytoplasm after lysosomal leakage caused by exogenous oxidants (reactive oxygen species) [73,74]. Acidification of the pericellular space of monocyte-derived macrophages, lung macrophages and osteoclasts

Table 3
Substrate specificity of cathepsin K

(a)

P3	P2	P1	P1′	P2′	Natural substrates
					Human collagen type I [109]
					N telopeptide domain
Gly	Ile	Ser	Val	Pro	α-1 chain
	Leu	Ser	Tyr	Gly	α-1 chain
Gly	Val	Gly	Leu	Gly	α-2 chain
					Helix cross-linking domain:
Gly	Pro	Arg	Gly	Asp	α-1 chain
Gly	Ile	Lys	Gly	His	α-1 chain
Gly	Pro	Arg	Gly	Leu	α-2 chain
Leu	Pro	Gly	Leu	Lys	α-2 chain
	Pro	Gln	Gly	Phe	Bovine collagen type I: α-1 and α-2 chains [106]
Lys	Pro	Gly	Lys	Ser	Bovine collagen type II: α-1 chain [104]
Tyr	Val	His	Leu	Arg	Human endostatin [271]
Ala	Pro	Gln	Gln	Glu	Human osteonectin [40]
Lys	Leu	Arg	Val	Lys	
					Bovine aggrecan [272]
Gly	Pro	Arg	Leu	Leu	Link protein domain
Ala	Phe	Gly	Ser	Gly	Link protein domain
Ala	Ile	Ser	Val	Glu	G1 domain
Ser	Val	Glu	Val	Ser	G1 domain
Ser	Val	Ser	Ile	Pro	G1 domain
Ala	Pro	Ser	Thr	Ala	G1 domain
					Thyroglobulin type 1 domain [244]
Lys	Leu	Ser	Lys	Gly	First loop of Testican
Ser	Leu	Leu	Gly	Ala	First loop of Testican
Leu	Leu	Gly	Ala	Phe	First loop of Testican
Arg	Ile	Gln	Lys	Leu	First loop of Testican
Pro	Pro	Gly	Phe	Ser	Kinin [172]
Lys	Ile	Lys	Phe	Asp	Apolipoprotein B-100 (LDL) [165]
Leu	Thr	Lys	Tyr	Ser	

(b)

P3	P2	P1	P1′	P2′	Synthetic substrates
Lys>Gly>His>Met>Arg>Ala	Leu>Ile>Pro>Val>Phe	Arg>Lys>Gln>Met>Thr			Coumarin derived peptides [48,55,273]
Lys>Arg>Leu>Ala	Leu>Ile>Val>Pro	Arg>Gln>Gly>Lys>His	Asn>Phe>Leu>Gly>Ser	Arg>Ser>Phe>Asn	FRET peptides [54]

enhances the release of cathepsin K to promote extracellular proteolysis [75,76]. An H^+-ATPase pump may be involved in the production of an acidic subcellular space by transferring protons from the cytoplasmic to the extracellular space [77,78].

Immunolocalization [13,79–81], *in situ* hybridization [82,83] and fluorescence microscope studies [84] have shown that cathepsin K is much more abundant in osteoclasts along the bone resorption lacunae than are cathepsins B, L and S [13,38,85,86]. Cathepsin K mRNA has been detected in a variety of tissues including bone, ovary, heart, placenta, lung, skeletal muscle, colon and small intestine [15].

High concentrations of cathepsin K have been found in osteoclasts, osteoclast-like cells (giant multinucleated cells) and also in synovial fibroblasts and in rheumatoid arthritic joints, which are involved in the pathological erosion of articular cartilage [38,85–87], and in epithelioid cells of organ systems like the lung and thyroid gland [88–90]. Cathepsin K is also found in aortic smooth muscle cells [31], macrophages [34], in bronchoalveolar fluids [91,92], and is secreted by macrophages [76], which could be of considerable importance for the remodeling of the extracellular matrix.

3. Physiological and pathophysiological implications

Cysteine cathepsins are primarily involved in the intracellular breakdown of proteins in lysosomes, where up to 50% of proteins are degraded [93,94]. The recent discovery of

cathepsins with a more restricted tissue distribution suggests that these enzymes are more than simple scavengers [53,95,96]. For instance, Tepel and colleagues have shown that the cathepsin K in thyroid epithelial cells is involved in the release of thyroid hormones (thyroxins) from thyroglobulin at neutral pH [90,97]. Unlike cathepsins F, L and S, cathepsin K is not involved in the presentation of antigens to major histocompatibility complex II (for review see [98]).

3.1. Bone turnover

Lysosomal cathepsin K is crucial for bone remodeling, particularly in the breakdown of the collagen network [95,99–102]. Bone is a highly stable fiber-reinforced, mineralized tissue, undergoing a constant remodeling process. Bone-forming cells are called osteoblasts while osteoclasts are involved in bone resorbing activities. The resorptive step depends on the concord action of specific proteases able to remove the organic matrix (predominantly fibrillar type I collagen) and the solubilization of the inorganic mineral component (hydroxyapatite) by production and secretion of acid by the osteoclasts [77]. Type I collagen is widely distributed and is the major structural protein in bone, where it accounts for 90% of the protein matrix. It consists of covalently cross-linked triple helices containing two α1(I) and one α2(I) chains. The remaining 10% consists of non-collagenous proteins like osteocalcin, osteonectin, osteopontin, fibronectin, thrombospondin and bone sialoprotein. Matrix degradation is mainly due to the activity of cathepsin K, which represents 98% of the total cysteine protease activity, and of collagenases of the MMP family [13,15,103,104]. Several peptidases, such as cathepsins D, E (aspartic proteases) as well as cathepsins B and L, were initially described to take part in osteoclastic bone resorption (for review see [105]). Cathepsin K, which is predominantly expressed in osteoclasts is a potent extracellular matrix degrading enzyme and play a critical role in osteoclast-mediated bone resorption [13,41]. In contrast to other cathepsins, cathepsin K is capable of cleaving triple-helical collagens, at multiple sites within the native triple helix, into small peptides (Table 3) [104,106]. The foremost features of those sites is the frequent presence of proline residues in the P2 position, in repetitive Gly-Pro-Xaa sequences of α(I) chains, where Xaa is mainly proline and/or 4-*trans*-L-hydroxyproline. None of the cleavage sites identified so far correspond to those of collagenases of the MMP family, of the neutrophil serine elastase, or to those of bacterial collagenases [104,107]. MMPs cleave interstitial collagens at distinct Gly-Ile/Leu bond to generate 1/4 and 3/4 fragments [108]. Cathepsin K also degrades type I collagen in its non-collagenous termini (N- and C-telopeptide regions, see Table 3) and releases cross-linked N- and C-telopeptides, which can be detected in urine and serum by immunoassays [109,110]. These fragments provide a responsive measure of osteoclast-mediated bone resorption [111,112]. The collagen cleaving activities of other cysteine cathepsins such as cathepsins B and L are restricted to these sites [15,113,114] with a lesser extent compared to cathepsin K. Although cathepsin L has been proposed to play a role in bone resorption, its expression level in osteoclasts remains relatively low compared to those of cathepsin K [13,15]. Cathepsin K also efficiently degrades osteonectin [40]. Inhibition of cathepsin K by specific inhibitors or by cathepsin K antisense oligonucleotides results in the accumulation of undigested collagen fibrils in lysosomes within osteoclasts [61,83,115,116]. The critical role of cathepsin K in bone remodeling is corroborated by the finding that deficiency in cathepsin K activity causes the autosomal recessive bone sclerosing disorder, pycnodysosotosis (see further sections). Its collagenolytic activity is specifically modulated by C4-S present in bone and cartilage, and requires the formation of a unique multimeric protein-glycosaminoglycan complex [117,118]. Recent studies have investigated the specificity of the binding interactions of cathepsin K to the negatively charged GAGs [119].

The regulation of osteoclastic bone resorption involves several factors such as cytokines (TNF-α and IFN-γ), hormones (estrogen and retinoic acid) and nuclear transcriptional factors. For instance, RANK-L, IL-1 (interleukin 1), IL-6, IL-11, IL-15 and IL-17, TNF-α and various transcription regulatory components (AP-1, MITF, PU.1) are necessary for osteoclastogenesis and bone resorption, whereas IFN-γ, as well as IL-10, IL-12, IL-13, IL-18, osteoprotegerin, calcitonin, and estradiol inhibit it (see for reviews: [120,121]). As described previously, they also regulate cathepsin K gene and protein expression [120,121]. RANK-L via the NF-κB signaling cascade participates in cathepsin K gene expression [36,37,122] and seems to be regulated by the combination of the transcription factors (NFATc1, PU.1 and MITF) and p38 MAP kinase [123,124].

3.2. Skeletal, heart, lung and intestinal development

Cathepsins K and L are both involved in the development of the skeleton, especially in that of long bone and the skull [128]. The respiratory and gastrointestinal mucosae of human embryos are both rich in cathepsin K [88]. It is also found in chronically inflamed gastric mucosae, suggesting its involvement in the processing of factors such as the release of pepsinogen [129]. Intense cathepsin K-specific immunoreactivity was found in the lumen of airways in early lung development, especially in bronchial epithelial cells. The enzyme could be involved, together with others proteases, in the rapid remodeling of lung tissue [130]. Some authors have reported finding cathepsin K in the endocardium in the NFATc-1 dependent RANKL signaling pathway, which is necessary for the development and morphogenesis of the heart valve leaflets [131], and in mouse aorta endothelial cells [132]. Cathepsin K together with cathepsins L and S, plays an important role in elastinolysis, since it can also cleave mature insoluble elastin, a structural component of the arterial wall that is notoriously resistant to proteolysis [15,31,95]. The interactions between elastin and cathepsins K, L and S have been explored in detail by Novinec et al. [125]. However, others have suggested that cathepsin V is a more potent elastase than cathepsin K, leukocyte elastase or cathepsin G [126]. Cathepsin K, which is present in dermal fibroblasts, may play a role in the homeostasis of

the dermal extracellular matrix during scar formation, while there is little cathepsin K in normal skin [127].

3.3. Reproduction

Secreted and lysosomal cathepsin K has been found, with cathepsin L, in the Sertoli cells of adult rat testes at stages VI–VII of spermatogenesis (in the lumen of the seminiferous epithelium). The authors suggest that these enzymes have overlapping functions in the breakdown of the residual body of Sertoli cells, facilitating germ cell movement and spermiation [133]. Cathepsins K and L knockout mice are both fertile, while the seminiferous tubules of mice lacking cathepsin L are atrophied [99,133,134]. The occurring temporal and spatial distributions of cathepsin K change during development and organogenesis, suggesting that their physiological roles vary in these cells.

3.4. Pycnodysostosis and osteoporosis

To date, two genetics disorders have been traced to the genes encoding cysteine cathepsins: Papillon–Lefevre syndrome, a periodontopathia caused by cathepsin C deficiency [135] and pycnodysostosis caused by cathepsin K deficiency [136]. Pycnodysostosis is a rare hereditary bone disorder in which osteoclast function in bone resorption is defective; victims suffer from osteosclerosis and short stature. This disease is linked to mutations in the gene encoding cathepsin K [136]. Osteoclasts from these patients accumulate undigested collagen fibrils [137]. Non-sense, mis-sense and stop codon mutations in the propeptide and the mature polypeptide have been identified in the patients [85,136,138,139]. Studies on cathepsin K-deficient mice have shown that they have a similar osteopetrotic phenotype due to lack of bone resorption [99,140]. Similar results were obtained with *in vitro* studies using antisense oligodeoxynucleotide against cathepsin K mRNA [116,141].

Several clues indicate that cathepsin K is also involved in bone resorption disorders like osteoporosis and in Paget's disease, since the osteoclasts at sites of extensive bone loss as well as serum with osteoclastic activity contained high concentrations of cathepsin K [4,83,142]. Transgenic mice that overproduce cathepsin K have a reduced trabecular bone volume as a result of increased bone resorption [143]. Its essential role in osteoclast-mediated collagen degradation is highlighted by the presence of N-telopeptide (NTx) collagen fragments in the urine/serum of human suffering from osteoporosis [112]. Treatment with a specific inhibitor of cathepsin K resulted in a significant decrease (relative to untreated controls) in serum markers of bone resorption, the reductions in N-terminal telopeptides (NTx) and C-terminal telopeptides (CTx) of type I collagen being 61% and 67%, respectively (estrogen-deficient monkey model of osteoporosis) [144]. A decrease in serum osteocalcin was also observed. As NTx are typical products of cathepsin K cleavage, they could be a sensitive marker for osteoporosis [109,145].

While caspases are the most prominent cysteine proteases involved in the initiation and execution of apoptosis, cathepsins B and L are also involved in cell death (see for reviews [73,96,146]). Cathepsins released into the cytoplasm by lysosomal permeabilization may initiate or enhance the activation of caspases [147–150]. A recent report indicates that cathepsin K deficiency mice (mimicking pycnodysostosis) lack normal apoptosis and senescence [151]. The authors highlight the key role of cathepsin K in osteoclast homeostasis by inducing either apoptosis in cat K−/− mouse strains with defective p19, p53 and p21 synthesis or senescence when cathepsin K is overproduced by pre-osteoclasts.

3.5. Rheumatoid arthritis and osteoarthritis

Victims of osteoarthritis (OA) and rheumatoid arthritis (RA) both suffer from the progressive destruction of articular cartilage, with the gradual loss of proteoglycans (aggrecan) and of collagen II (for review see [4]). Cartilage is a tough, elastic, fibrous specialized connective tissue found in various parts of the body, such as the joints. Cartilage tissue consists of a relatively small number of cells and an abundant extracellular matrix. Fibrillar type II collagen is the major component of the extracellular matrix of articular cartilage, forming a 3-D network of fibrils which gives the tissue its tensile strength. Under physiological conditions, type II collagen is highly resistant against proteolytic degradation. Hydrophilic aggrecans are the second main component, which are responsible for the compressive stiffness of cartilage [152]. Degradation or loss of either one or both molecules results in the destruction of articular cartilage [153]. Metalloproteases such as collagenases (MMP-1, MMP-13) and aggrecanases have been implicated in the destruction of the cartilage matrix (for reviews see [3,4]). However, increasing evidence suggests a critical role of cysteine cathepsins in the pathogenesis of RA as well. Cathepsin K is know to play a role in osteoarthritis [101,154,155]. Cathepsin K activity increases as collagen is broken down and its endogenous inhibitor (cystatin C), the most abundant extracellular inhibitor of cysteine proteases, is overwhelmed [156–159].

Cathepsin K is present in synovial fibroblasts, where it plays a critical role in the pathogenesis of RA by breaking down aggrecans and type II collagen, leading to joint destruction [85]. Cathepsins S and K are both powerful enzymes in the breakdown of aggrecans at low pH [85]. Aggrecan have protein backbone with three globular domains (G1–G3) covalently bound to chondroitin and keratan sulfates side chains. Cathepsin K cleaves sites in the G1 domain, the two interglobular domains E1 and E2 and the link protein (Table 3). In contrast to the hydrolysis of aggrecan, the degradation of type II collagen is restricted to activities of collagenolytic MMPs and cathepsin K [104]. Cathepsin K is involved in the hydrolysis of these substrates (aggrecan and collagen) by forming active proteolytic complexes with chondroitin or keratan sulfates (GAGs) [117,118,160]. In contrast, GAGs reduce the collagenolytic activity of cathepsins L and S, providing a novel mechanism of cathepsin regulation. A recent study on a cohort

of patients suffering from longstanding rheumatoid arthritis (RA) found a correlation between rheumatoid arthritis and an increase in serum cathepsin K, suggesting that cathepsin K may become a new marker of RA [161].

3.6. Atherosclerosis—Cardiovascular diseases

Abnormally high cathepsin K production and the massive destruction of elastin or collagen are features of several clinical situations, such as vascular inflammation (arteritis), causing aneurisms of the abdominal aorta. Patients with severe vascular disease also have enhanced elastinolytic activity, increased amounts of cathepsins K and S protein and mRNA during the formation of new intima, and subnormal amounts of cystatin C [162]. Cathepsins secreted by smooth muscle cells or macrophages into the intimal matrix might act on low-density lipoprotein particles, particularly those in apolipoprotein (apo) B-100, to form the potentially cytotoxic lipid deposits, responsible for atherosclerosis [163]. Extensive investigations, especially studies those on mice with targeted gene deletion (simple knock-out of cathepsin K or double knock-out of cathepsin K and apolipoprotein E), have demonstrated the pathophysiological relevance of cathepsin K in vascular remodeling and atherosclerosis [164]. Linke and colleagues identified sites cleaved by cysteine cathepsins in apo B-100 that lead to changes in the structure of LDL particles. Thus cathepsins may be involved in the progression of atherosclerosis [165]. The transient synthesis of cathepsin K might also be regulated by shear-stress in endothelial cells, favoring the remodeling of arteries [132]. Secreted cathepsin L may act similarly, underlining the potential role of cathepsins in cardiovascular diseases [166,167].

3.7. Lung inflammation

Cysteine cathepsins may be critical in the homeostasis of deposition and recycling of ECM proteins that occurs in chronic inflammatory lung diseases (emphysema, pulmonary fibrosis). The lung ECM is destroyed by proteolysis in emphysema, while matrix deposition by activated fibroblasts leads to pulmonary fibrosis. The lungs of transgenic mice that mimic COPD with emphysema caused by inducible cytokines IL-13 and IFN-γ have increased production of MMPs and cathepsins B, S, L, H and K [32,33]. The cathepsin K produced and secreted by macrophages, smooth muscle cells and fibroblasts may degrade elastin. In contrast, inhibiting the cysteine cathepsins in human fibroblasts or administering bleomycin to fibroblasts from cathepsin K(−/−) mice lead to increase matrix deposition, suggesting that the enzyme is pivotal in lung fibrosis [168]. We have identified the proform and mature forms of cysteine cathepsins in the bronchoalveolar fluids of patients suffering from silicosis or sarcoidosis [91,92,150]. The ratio between proteases and cystatin-like inhibitors is skewed in favor of proteolysis, providing yet more evidence of the role of this system in the breakdown and/or remodeling of the ECM.

Bradykinin (BK, sequence: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and related kinins (such as lysyl-bradykinin), are peptide hormones whose pharmacological effects are mediated either by inducible (B1-type) or constitutive (B2-type) kinin receptors. They help maintain cardiovascular homeostasis; they are also implicated in inflammatory disorders and may induce bronchoconstriction and airway hyper-responsiveness in asthmatic patients (for review see [169]). Kinins are generally produced from kininogens by tissue and plasma kallikreins, but there may be alternative pathways [170]. The cysteine cathepsins, cathepsins L and K may be involved in kinin metabolism. While cathepsin L has kininogenase activity [171–173], cathepsin K is the only mammalian cysteine protease that exhibits a potent kininase activity *in vitro* (scissile bond: Gly–Phe) [174], in addition to the peptidases, NEP (neutral endopeptidase) and ACE (angiotensin-converting enzyme) [169]. Furthermore, cathepsin K modulates the bradykinin-dependent contraction of isolated bronchial smooth muscles, and weakens the kinin-induced drop in blood pressure in rats [175].

3.8. Cancer

While clinical trials with MMP inhibitors to treat cancers and arthritis have led to conflicting results, cysteine cathepsins have become interesting targets for drug (for review see [6]). There is now good evidence that cysteine cathepsins, especially cathepsins B, L, H, S, X and K, contribute to stroma degradation and angiogenesis during tumor progression and thus promote the growth and metastasis of tumor cells [176–178]. Transcription regulators like ETS-1, which is produced by invasive tumors and binds to the 5′ region of the cathepsin K gene promoter, may regulate cathepsin K synthesis in tumors and tumor-associated cells. Reports indicate that human cathepsin K is associated with increased invasive potential by prostate cancers, thyroid carcinoma, lung adenocarcinoma, breast carcinomas, and by several breast cancer cell lines [179–184].

3.9. Miscellaneous

3.9.1. Amyloidosis

Amyloidosis is characterized by the deposition of amyloid proteins in tissues. Local deposits of amyloid are frequent in the brains of patients with the Alzheimer's disease. Cathepsin K, which is associated with amyloid deposits, can degrade native amyloid fibril and precursor proteins *in vitro* [185,186]. Cathepsin K (−/−) mice had over 90% more amyloid deposits than the controls, unlike cathepsins B and L, suggesting that cathepsin K is involved in the modulation of amyloid deposits in this disease [187].

3.9.2. Gaucher disease

Gaucher's disease is a rare lysosomal storage disease in which glucocerebrosides accumulate in macrophages. This leads to massive enlargements of the spleen and liver, bone infarction crises, osteolytic lesions, and tissue damage in the liver, lung, bone marrow, and brain stem. The activities of cathepsins K, B, and S are abnormally high in the plasma and

spleen of these patients [188]. However, the relevance of these observations remains unclear.

3.9.3. Obesity

The morphology of adipocytes cells changes dramatically and the pattern of their gene expression is altered in obese subjects. There is a strong correlation between the obese subject's body mass index and the overexpression of the cathepsin S gene and the high circulating concentration of the enzyme. As cathepsin S has a great elastinolytic activity and may be involved in the development of atherosclerotic lesions, it may be a promising target for attempts to reduce cardiovascular risks in obese patients [189–191]. The production of cathepsin K is also enhanced in mouse models of obesity and in the adipose tissue of obese patients, and this increase is significantly correlated with other markers of increased adiposity [23]. Its role in the remodeling of the ECM and basement membrane (osteonectin, collagen IV) may contribute to the process of adipose mass enlargement.

4. Regulation of cathepsin K activity

4.1. pH

It has been known for decades that the activities of cysteine cathepsins are optimal under slightly acid conditions. With the noticeable exception of cathepsin S, human cathepsins were believed to be rapidly inactivated at neutral pH or under alkaline conditions [192,193]. Nevertheless cathepsin K revealed a relative time-dependent stability at pH 7.5 and at 28 °C (≈59% of residual activities after 30 min of incubation, 31% after 60 min and 8% after 120 min) [48]. Conversely, cathepsins are inactivated irreversibly at acidic pH; this inactivation is accompanied by large structural changes and a loss of alpha-helix content [194]. However, the binding of substrates or inhibitors to mature cysteine cathepsins, may partly prevent their inactivation [194]. Human cathepsin K has a broad bell-shaped pH activity profile with an optimum at 6.0 which allows full activity within the lysosomal compartment as well as for solubilizing bone minerals [48,195].

4.2. Propeptide

The propeptide is a feature of all cysteine cathepsins. It is responsible for the proper targeting, folding and stability of cathepsin K. The proregion is also a selective competitive inhibitor of its parent enzyme (reviews are recommended for interested readers: [196–198]). This is because the propeptide can cover the active site cleft in a non-productive orientation that does not allow the hydrolysis of a peptide bond [199,200]. The cathepsin K propeptide inhibits cathepsins S and L as well as its own mature enzyme (K_i in the nanomolar range: 2–5 nM), indicating that it is poorly selective towards these related enzymes. In contrast, the cathepsin K propeptide poorly inhibits cathepsin B (>400 nM) [201,202]. Crystal structures of procathepsin K indicate that the residues Lys^{79p}-Leu-Gly-Thr-Met^{75p} (procathepsin K numbering) cover the active site cleft (respectively S3–S2′ subsites), with Leu^{78p} in the S2 pocket. This sequence may prove to be valuable for the development and design of highly selective proregion-derived inhibitors, as reported for human cathepsin L [203].

4.3. Glycosaminoglycans

The endopeptidase activity of cathepsin B is potentiated by heparin and heparan sulfate—they reduce the loss of α-helix content at neutral pH [72,204]. The negatively charged chondroitin 4-sulfate, which constitutes 90% of the total glycosaminoglycan content in bone [205], stabilizes cathepsin K by forming active complexes and potentiates its collagenolytic activity towards soluble and insoluble type-I and II collagens, while it reduces that of cathepsins L and S [118,206]. On the other hand, chondroitin 6-sulfate, dermatan sulfate and heparan sulfate have no effects. Brömme and coworkers reported the formation of a specific oligomeric cathepsin K/C4-S active complex [118]. Electrostatic surface potential maps reveal clusters of positively charged arginine and lysine residues (opposite to the binding cleft of the protease, see Fig. 1). The existence of an additional interaction site offers a new target and a novel approach to the inhibition of excessive bone degradation without affecting the proteolytic function of the monomeric enzyme. Thus, the capacity of negatively charged polyamino acids like poly-L-Asp, poly-L-Glu to prevent the formation of the complex by competing with the sulfate groups of C4-S has been tested recently [119]. These polypeptides can inhibit the attachment of C4-S to collagen and also prevent proteolysis by masking the cleavage sites in collagen.

4.4. Oxidation

Some effects of reactive oxygen species (ROS) on cell metabolism and particularly on proteases have been recently documented (for review see [207]). Inadequate control of ROS takes part in the initiation and pathophysiology of OA, contributing ultimately to an imbalance between proteases and inhibitors or oxidants and antioxidants, and increased apoptosis (see for reviews [208–210]). ROS also stimulate inflammatory pathways and may damage lipids, proteins and DNA. However, findings on the anti-inflammatory properties of ROS in articular cartilage are conflicting [211]. Percival and coworkers have shown that cathepsin K activity is inhibited by reactive species (nitric oxide, S-nitroso derivatives of glutathione, *N*-acetylpenicillamine, NO donors) *in vitro*, in a time and concentration-dependent manner, via different oxidation states of Cys^{25} [212].

We identified proforms and active cysteine cathepsins in the bronchoalveolar lavage fluids of patients with acute inflammations or silicosis, despite the presence of an unfavorable oxidizing environment [91,92], indicating that the lung has effective antioxidant systems to protect it from oxidants. We demonstrated that the proteolytic activity of cathepsin K is partially conserved under supraphysiological GSH/GSSG balance conditions *in vitro*. C4-S can also protect the enzyme against inactivation by oxidized glutathione (E. Godat, V.

Hervé-Grépinet, F. Veillard, F. Lecaille, M. Belghazi, D. Brömme, G. Lalmanach, unpublished data).

5. Natural inhibitors of cathepsin K

Cysteine protease inhibitors with appropriate pharmacological properties could be designed using endogenous protein inhibitors as a framework. Surprisingly, few reports have focused on natural inhibitors (i.e. the cystatins superfamily) of cathepsin K. Studies on the mechanism of action and the binding of these inhibitors would provide valuable information for developing drugs to treat human diseases [213]. Recently, a comprehensive classification of protease inhibitors has been developed in the MEROPS database (http://merops.sanger.ac.uk). Dubin has reviewed the proteinaceous cysteine protease inhibitors identified to date [214]. The following section is an overview of our current knowledge on the endogenous natural inhibitors of cathepsin K that are widely distributed in humans. We also include unpublished K_i values of cathepsin K with cystatins (i.e. stefin B, cystatin C, the H- and L-kininogens) (Table 4).

5.1. The cystatin superfamily

The cystatin family (family I25; clan IH) is divided into three groups (stefins, cystatins and kininogens) based on their structure, distribution in the body and physiological roles. Stefins and cystatins are very stable molecules even at extreme pH and high temperature. The structures of a few enzyme–inhibitor complexes are available, including those of chicken egg white cystatin–papain [215], stefin B–papain [216] and stefin A–cathepsin H [217]. X-ray crystallography and NMR spectroscopy studies of cystatins (chicken cystatin: PDB code 1CEW, stefin A: 1DVD and stefin B: 1STF) indicate that they are all similarly folded, suggesting that the interactions of all cystatins are the same and involves three conserved regions (see for reviews [218,219]). The first is the N-terminal glycine-containing region (Gly^{11} according to human cystatin C numbering). The second segment is a central pentamer (Gln^{55}-Xaa-Val-Xaa-Gly^{59}), which stabilizes the complex via an area of extended contact with the proteinase. The third segment has the conserved Pro^{105}–Trp^{106} (or His/Gly^{106} in type I cystatin) pair. The cystatins are reversible, tight-binding competitive inhibitors of cysteine proteases. Bode et al. [220] have proposed that the S3-S2-S1 subsites of cysteine cathepsins accommodate, in a substrate-like manner, the side chains of the residues in positions 9, 10 and 11 (Ile-Pro-Gly for stefin A; Met-Cys-Gly for stefin B; Leu-Val-Gly for cystatin C) of the N-terminal segment of the cystatin, while subsite S1′ interacts with the second segment and subsite S2′ with the third.

5.1.1. Stefins (family 1)

Type 1 cystatins are polypeptides with a molecular weight of about 11 kDa, encoded on chromosome 21, and have no disulfide bonds and no carbohydrate side chain. Stefin A (cystatin A; p*I*: 4.5–4.7; MEROPS id I25.001) and stefin B (cystatin B; p*I*: 6.0–6.6; MEROPS id I25.003) are acidic proteins that are mainly found in intracellular compartments [219]. Stefin A is restricted to the skin and some blood cells in mammals, suggesting that its main role is to defend the body against cysteine proteases produced by pathogens [221]. Stefin B is widely distributed in almost all cells and tissues (liver, spleen, placenta, epithelial cells) and is considered to be a general cytosolic inhibitor, probably protecting the cell against uncontrolled proteolysis by cathepsins. An imbalance between cathepsins and stefins in favor of proteolysis contributes to the development of the invasive cell phenotype and to tumorigenicity or metastasis [222]. Lack of stefin B synthesis is characterized by a progressive myoclonus epilepsy [223]. Stefin B may help regulate bone resorption by blocking cathepsin K activity in osteoclasts, as reported for type 2 cystatins

Table 4
K_i values for the inhibition of human wild type cathepsins K and L and their respective S2 subsite mutants (L67Y/A205L cathepsin L and Y67L/L205A cathepsin K) by cystatins

Enzymes	K_i (pM)			
	Stefin B	Cystatin C	L-kininogen	H-kininogen
	(family 1)	(family 2)	(family 3)	(family 3)
wt cathepsin K	12.4 ± 6.6	4.1 ± 1.5 (2.8[a])	4.9 ± 1.6	5.4 ± 0.6
+C4-S (0.15%)	n.d.	n.d.	5.3 ± 2.0	15.0 ± 2.1
cat K (Y67L/L205A)	0.8 ± 0.1	8.7 ± 1.0	13.5 ± 5.0	14.5 ± 6.0
wt cathepsin L	9.5 ± 3.5 (9[b])	6.3 ± 0.3 (5.0[c])	13.9 ± 3.8 (17[d])	12.9 ± 1.2 (19[e])
+C4-S (0.15%)	n.d.	n.d.	13.3 ± 2.6	15.3 ± 2.0
cat L (L67Y/A205L)	6.3 ± 0.7	3.9 ± 0.2	2.6 ± 0.4	3.9 ± 1.5

Inhibition constants were determined working under experimental conditions such that non linear dose-response curves were obtained by using the Easson and Stedman procedure [274]. Enzyme concentrations [E_0] were at least 1–10 higher than K_i values and less than 5% of the substrate (Z-Phe-Arg-AMC) was hydrolyzed during the progress of the experiments ($\lambda_{ex} = 350$ nm; $\lambda_{em} = 460$ nm). Assays were performed at 30 °C in 0.1 M sodium acetate buffer, pH 5.5, containing DTT 2 mM, EDTA 2 mM. Data were reported as means ± S.D. ($n = 3$). n.d., not determined.

[a] From Brage et al. [275].
[b] From Lenarcic et al. [276].
[c] From Abrahamson et al. [277].
[d] From Salvesen et al. [278].
[e] From Muller-Esterl [233].

[158,224]. Human recombinant stefin B has similar K_i values for cathepsins K (12.4 pM) and L (9.5 pM) (Table 4). We have developed two mutants of cathepsins K and L by exchanging two residues of their S2 subsites [48,57]. The affinity of the cathepsin L-like cathepsin K mutant was 10-fold lower than that of wild-type cathepsin K, suggesting that Tyr^{67} and Leu^{205} may be critical for interaction with stefin B. Conversely, the cathepsin L $Leu^{67}Tyr/Ala^{205}Leu$ mutant ($K_i = 6.3$ pM) and its parent enzyme ($K_i = 9.5$ pM) had similar K_i value.

5.1.2. Cystatins (family 2)

Most cystatins are non glycosylated single chain proteins with molecular masses of 13–14 kDa. Unlike stefins, they contain two intramolecular disulfide loops at the C-terminus. They are mainly extracellular, in all body fluids at significant high concentrations [225]. Cystatins C, D, S, SA and SN are related to this family, with more than 50% amino acid sequence identity. Cystatin C has been thoroughly studied; it is ubiquitous in all extracellular fluids, while cystatins D, S, SA and SN are mainly found in saliva [226]. Glycosylated cystatins E/M were recently identified in the skin epithelium and glycosylated cystatin F was found in hematopoietic cells [226]. It has been suggested that cystatin M may suppress breast cancer tumors [227]. Cystatin C is the tightest-binding inhibitor of cysteine proteases [43,226, 228,229]. It inhibits cathepsins in the picomolar range (cathepsin K, $K_i = 4.1$ pM; cathepsin L, $K_i = 9.5$ pM; cathepsin S, $K_i = 8.0$ pM and cathepsin V, $K_i = 20$ pM) [47,230].

High concentrations of cathepsins B, H, K, L and S have been detected in the lung tissues of baboons with bronchopulmonary dysplasia, while the steady state mRNA and protein concentrations of stefin B and cystatin C were unchanged [231]. Similar results have been reported for atherosclerosis, where there was a reduction in or a lack of cystatin C protein along with extensive remodeling of the ECM (arterial wall). This could be an alternative to increase protease activity in the development of inflammatory disease (for review see [232]).

5.1.3. Kininogens (family 3)

There are two glycosylated forms of human kininogens that differ in the length of their C-terminal regions. They are designated H-kininogen (HMWK; high molecular weight kininogen; MW ca. 120 kDa) and L-kininogen (LMWK; MW 50–80 kDa) (for reviews see [233,234]). These multi-domain proteins are synthesized in the liver and found in the blood plasma, where α_2-macroglobulin and the kininogens are the major inhibitors of cysteine cathepsins. Both L- and H-kininogens harbor three tandemly repeated type 2-cystatin domains in their N-terminus (D1, D2 and D3), a short domain D4 (corresponding to the 9-mer BK), and a light chain (one domain D5 for LMWK, and 2 domains D5/D6 for HMWK). Only domains 2 and 3 are functionally active inhibitors of cysteine proteases. The two kininogens have similar K_i values for cathepsin K, approximately 2-fold lower than that of cathepsin L (Table 4). As the collagenolytic activity of cathepsin K is specifically mediated by negatively charged C4-S, we measured its inhibition in the presence of C4-S. The K_i values were similar to those of monomeric cathepsin K (~5 pM), despite a slight increase with H-kininogen (~15 pM). Thus C4-S does not affect inhibition by kininogens.

5.1.4. Cystatin-derived inhibitors

Diazomethyl peptides derived from the substrate-like region of cystatin C have been designed to inhibit cysteine proteases [235,236]. This framework was used to develop an activity-based probe by adding a biotin group to the N-terminus [237]. However, the probe is poorly selective, since it targets not only cathepsins B, H, L, S, and cathepsin K [91], but also trypanosomal cysteine cathepsins [238] or falcipains from *Plasmodium falciparum* [239]. Peptidomimetic inhibitors (azapeptides) of cysteine proteases were designed by replacing Gly^{11} by an azaglycine (α-CH is replaced by a nitrogen atom) [240,241]. These compounds are good inhibitors of cathepsins B and K. However, selective azapeptidyl inhibitors have been obtained for cathepsin B, but not for cathepsin K.

5.2. Other inhibitors

5.2.1. Thyropins (thyroglobulin type-1 domain proteinase inhibitors)

The thyropins include saxiphilin, equistatin, testican, chum salmon egg cysteine protease inhibitor (ECI), IGFBPs and p41 invariant chain fragment (see for review [242]). Thyropins bind reversibly and tightly to cysteine cathepsins. Studies on the crystal structure of p41-invariant chain fragment in complex with cathepsin L revealed the mode of inhibition [243]. It does not inhibit cathepsin K, but cathepsin K can degrade the thyroglobulin type-1 (Tg-1) domain of testican [244]. Modeling studies suggest that steric constraints prevent the Tg-1 domain reaching the active site of the enzyme.

5.2.2. Serpins (serine proteinase inhibitors)

Most inhibitory serpins are proteins of about 45 kDa which target serine proteases, including thrombin, trypsin and human neutrophil elastase, via a highly exposed flexible reactive site loop (RSL) (see for reviews [245,246]). Some serpins that inhibit other classes of protease (cysteine, aspartic and metallo proteinase) are termed "cross class inhibitors" and act by suicide substrate-like inhibition. Human serpin squamous cell carcinoma antigen-1 (SCCA-1: serpinB3), the avian serpin myeloid and erythroid nuclear termination stage specific protein (MENT) and the human serpin headpin (serpinB13) all inhibit cathepsins K, L and S by forming tight complexes at a stoichiometry of 1:1 [247–249]. SCCA-1 is present in the sera of cancer patients and is a potent inhibitor of cathepsin K, with second order rate constants (k_{ass}) comparable to that of cystatin C [247]. Although SCCA-1 is nearly identical to SCCA-2, a homologous squamous cell carcinoma antigen (91% of identical amino acids), it does not inhibit serine proteases [250]. Besides the importance of the RSL, the residue at the P2 position may serve as an anchor for inhibitor-protease interactions. The low concentration of serpin in some cancer

cell lines, together with increased concentrations of cathepsins K and L, may affect the development of metastasis [251,252].

5.2.3. α_2-Macroglobulin

Human plasma α_2-macroglobulin (α2-M) is a tetramer of 4 identical 185-kDa subunits; it can inactivate serine-, cysteine-, aspartic- and metalloproteinases (for review see [253]). The cathepsins B, H and L all become irreversibly entrapped within the tetramer so that they are unable to hydrolyze large substrates because of steric hindrance, but they retain their ability to cleave small proteins (10–20 kDa) [254,255]. No studies on the inactivation of cathepsin K by α_2-macroglobulin have been published to date.

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