Human and Parasitic Papain-Like Cysteine Proteases: Their Role in Physiology and Pathology and Recent Developments in Inhibitor Design

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1. Introduction

Proteases can be categorized based on their substrate specificities or mechanisms of catalysis. Enzymes cleaving within a polypeptide chain are named endopeptidases, and activities cleaving at the ends of polypeptides are named exopeptidases. Aminopeptidases cleave at the N-terminus and carboxypeptidases cleave at the C-terminus of substrates. Upon the basis of the mechanism of peptide hydrolysis, five major protease classes are known: serine, cysteine, aspartic, threonine, and metallo-proteases. This review will focus on papain-like cysteine proteases, the largest subfamily among the cysteine protease class (clan CA, family C1). Papain-like cysteine proteases are widely expressed throughout the animal and plant kingdoms and have also been identified in viruses and bacteria. Mammalian papain-like cysteine proteases are also known as thiol-dependent cathepsins. From a pharmaceutical point of view, these proteases have obtained little attention since it was thought that their functions are restricted to the nonspecific degradation of proteins in lysosomes or equivalent cellular compartments. Recent progress in the understanding of the physiological roles of these enzymes using protease-deficient mouse models has changed this view. It becomes more and more transparent that papain-like cysteine proteases fulfill specific functions in extracellular matrix turnover, antigen presentation, and processing events and that they may represent viable drug targets for major diseases such as osteoporosis, arthritis, immune-related diseases, atherosclerosis, cancer, and for a wide variety of parasitic infections. This review will summarize the biochemistry of human and parasite cysteine proteases and recent developments in inhibitors design, and will focus on their role in physiology and pathology.

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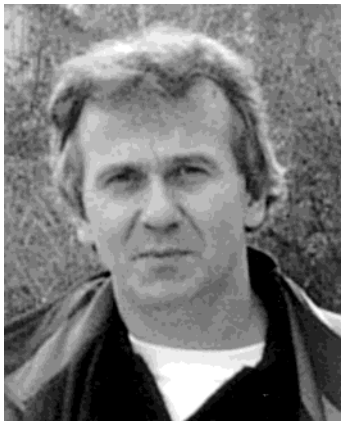
Fabien Lecaille was born in 1972 and obtained his Ph.D. in Biochemistry (2000) at the Francois Rabelais University in Tours (France). His work focused on the regulation of parasite cysteine proteases by peptidyl substrates and inhibitors under the guidance of Dr. Gilles Lalmanach. Since 2001, he has been working with Dr. Dieter Brömme on human cathepsin K collagenase activity.

Jadwiga Kaleta obtained her M.Sc. in Biological Sciences, Specialization in Biochemistry from the University of Lodz (Poland) in 1975. Before immigrating to Canada in 1981, she worked at the Polish Academy of Sciences in a field of biogenic amines, gaining experience in neurotransmitter studies and their pharmacological applications. In Canada, she joined SynPhar Laboratories in Edmonton, which became NAEJA Pharmaceutical in 1999, and is presently Associate Director of Enzymology with a focus on assay developments for bacterial, fungal, and mammalian enzymes. Her main interest is directed to the utilization of small molecular weight inhibitors for proteases, in particular for cysteine proteases.



Dieter Brömme, born in 1955, studied Biochemistry from 1975 to 1980 at the Martin-Luther-University of Halle (GDR). In 1983, he received his Ph.D. under the guidance of Prof. Dr. Rolf Kleine from the same university. In 1991, he joined the Biotechnology Research Institute (NRC) in Montreal (Canada) and moved in 1993 to California to join a Biotech Startup company (Khepri Pharmaceuticals; South San Francisco). In 1997, he returned to academia as an Associate Professor of Human Genetics at the Mount Sinai School of Medicine in New York City. His main interests are human papain-like cysteine proteases and their roles in health and disease.

2. Papain-Like Cysteine Proteases: Structure, Mechanism, Expression, Substrate Specificity, and Physiological Functions

2.1. Amino Acid Sequences and Evolutionary Relatedness

On the basis of the human genome database, 11 papain-like cathepsins are expressed in the human genome. All of them had been cloned and at least partially characterized before the completion of the sequencing of the human genome. Several dozens of cysteine proteases have been identified in various parasitic organisms, and their number is steadily growing. All papain-like cysteine proteases have in common that they consist of a signal peptide, a propeptide, and a catalytic domain with the latter representing the mature proteolytically active enzyme. Table 1 summarizes the length of the protease domains and their appropriate calculated molecular weights and isoelectric points as well as the chromosomal localization of the human genes. Signal peptides which are responsible for the translocation into the endoplasmic reticulum during ribosomal protein expression are on average between 10 and 20 amino acids in length. Propeptides are of variable length between 36 amino acids in human cathepsin X and 315 amino acids in falcipain-1, a major cysteine protease of the malaria causing parasite *Plasmodium falciparum*. Functions of the prodomains are at least 3-fold:[1] (i) the proregion acts as a scaffold for protein folding of the catalytic domain;[2,3] (ii) the prodomain operates as a chaperone for the transport of the proenzyme to the endosomal-lysosomal compartment;[4,5] and (iii) the proregion acts as a high-affinity reversible inhibitor preventing the premature activation of the catalytic domain.[6−9] The inhibitory activity of the propeptide reveals some specificity to the parent enzyme. However, available data indicate that the specificity is restricted to members of the cathepsin subfamilies. For example, the propeptide of cathepsin B is selective for cathepsin B and by orders of magnitude less effective for cathepsin L-like proteases.[6] On the other hand, propeptides from cathepsin L-like proteases show strong overlapping inhibitory activity within the protease subfamily.[10,11] The structure of cathepsin L-like propeptides appears to be stabilized by a tripartite tryptophan motif. Mutations of any of these tryptophane residues lead to a dramatic decrease of the inhibitory capacity of the respective propeptides.[12]

The catalytic domains of papain-like cysteine proteases are between 220 and 260 amino acids in length. Exceptions are several parasite-derived cysteine proteases which contain C-terminal extension of unknown function. The highest conservation among the three polypeptide domains is observed in the catalytic domain. Figure 1 shows the amino acid alignment of 11 human cathepsins and 49 parasitic cathepsins of major human and animal pathogens. All cysteine proteases have in common a conserved

active site consisting of a cysteine, histidine, and asparagine residue. The cysteine residue (Cys^{25} based on papain numbering) is embedded in a highly conserved peptide sequence, CGS**C**WAFS (active site cysteine residue in bold). Only a relatively small number of cysteine proteases have amino acid replacements in this region. Similar to the area around the active site cysteine residues also the vicinities of the histidine and asparagine residues are conserved. The histidine residue (His^{159}; papain numbering) is adjacent to small amino acid residues such as glycine or alanine followed by four aliphatic hydrophobic residues (valine, leucine, isoleucine) and a glycine. Asparagine (Asn^{175}; papain numbering) is part of the Asn-Ser-Trp motif. In addition to the active site residues, several other amino acid sequence regions show a high degree of conservation. Among those are several cysteine residues which are involved in the formation of disulfide bridges, Pro^{2} which might prevent the N-terminal truncation and thus inactivation of the mature protease by aminopeptidases, the Gly-Cys-X-Gly-Gly motif forming a wall along the nonprime subsite binding area and the Gly-Pro motif separating a β- and α-domain at the interface between the major L and R domains.

The overall three-dimensional fold of cathepsins is highly conserved. The structure consists of an L and R domain of similar size where the active site cysteine residue is located in a structurally conserved α-helix of the L-domain whereas the histidine residue is in the R-domain (Figure 2). The propeptide is less structured and runs in inverse orientation through the substrate binding cleft (Figure 2). A comprehensive review of cathepsin structures has been recently published by McGrath.[13]

Protein sequence alignments of human and parasitic cysteine protease catalytic domains reveal a clustering into three major subfamilies: cathepsin B-like, cathepsin L-like, and cathepsin F-like (Figure 3). The clustering is based on the general degree of sequence identity and similarity among the proteases. In addition, a typical feature of cathepsin B-like cysteine proteases is the insertion of a so-called occluding loop between the conserved $Pro\text{-}Tyr^{103}$ motif and Cys^{128} (cathepsin B numbering). The loop is characterized by two adjacent histidine residues (His^{110}, His^{111}) which are responsible for the dipeptidyl carboxypeptidase activity of cathepsin B.[14] Both histidine residues anchor the C-terminal carboxyl group of peptide and protein substrates and thus allow the proteolytic removal of C-terminal dipeptides.

The relatedness of the three subfamilies is also reflected in a conserved motif within the proregions which have a significant lower degree of sequence similarity than the catalytic domains. Karrer et al. identified the so-called ERF/WNIN motif as characteristic for the cathepsin L-like subfamily.[15] This motif is absent in cathepsin B-like proteases and modified into an ERFNAQ/A motif in the cathepsin F-like subfamily.[16,17] All of the proteases marked as cathepsin F-like in Figure 3 possess the ERFNAQ/A motif. Several cysteine proteases, among them the human cathepsins C, O, and X, do not allow a clear classification into one of the subfamilies. They may represent additional subfamilies.

2.2. Catalytic Mechanism of Cathepsins

The catalytic site of papain-like cysteine proteases is highly conserved and formed by three residues: Cys^{25}, His^{159}, and Asn^{175} (papain numbering). Cys^{25} and His^{159} form an ion pair which is stabilized by Asn^{175} via a hydrogen bond. This triad has some similarities to the active site present in serine proteases (Ser, His, Asp). However, in contrast to serine proteases the nucleophilic cysteine residue is already ionized prior to substrate binding and thus cysteine proteases can be regarded as a priori activated enzymes.[18] During peptide hydrolysis, the nucleophilic thiolate cysteine attacks the carbonyl carbon of the scissile bond of the bound substrate and forms a tetrahedral intermediate which is stabilized by the so-called oxyanion hole (for more details see review, ref 19). The tetrahedral intermediate transforms into an acyl enzyme (enzyme−substrate thiol ester) with the simultaneous release of the C-terminal portion of the substrate (acylation). This step is followed by the hydrolysis of the acyl enzyme with water, forming a second tetrahedral intermediate which finally splits into the free enzyme and the N-terminal portion of the substrate (deacylation). The sequence of the reaction is shown in Figure 4. The catalytic mechanism has been recently refined by proton inventory experiments.[20] Kinetic studies of solvent isotope effects suggest that the initial enzyme substrate complex already represents a tetrahedral adduct and that the basicity of the nitrogen atom of the scissile bond promotes the formation of the acyl enzyme. Furthermore, it is thought that proton transfers in acylation (from the imidazolium cation onto the nitrogen of the scissile bond) and in deacylation (from the nucleophile water molecule to the acyl enzyme), respectively, are concerted and not stepwise actions.

The catalytic site is located within the substrate binding region which has binding pockets (subsites) for substrate amino acids in N- and C-terminal direction from the scissile bond. According to the Schechter and Berger nomenclature,[21] subsites in N-terminal direction are named S1, S2, S3...Sn, and subsites in C-terminal direction are called S1′, S2′, S3′...Sn′. The appropriate substrate (or inhibitor) amino acids binding in the subsites are designated with P1, P2, P3, etc. and P1′, P2′, P3′, etc.

2.3. Intracellular and Tissue Distribution, Substrate Specificity, and Physiological Functions

Papain-like cysteine proteases are expressed as preproenzymes and are synthesized at the rough endoplasmic reticulum. They possess N-glycosylation sites which are utilized for the translocation to the lysosomal compartment via the mannose 6-phosphate receptor pathway. The location of papain-like cysteine proteases is not strictly lysosomal but rather the enzymes are trafficking between phagosomes, endosomes, and lysosomes and individual proteases may accumulate in different organelles. For example,

Table 1. Mammalian and Parasite Cysteine Proteases

proteases AC number (Swiss-Prot)	signal peptide AA	prodomain length AA	prodomain size Da	prodomain pI	mature domain length AA	mature domain size Da	mature domain pI	prepro-enzyme length AA	prepro-enzyme size Da	chromosomal position
Human Cathepsins										
cathepsin L P07711	17	96	11724	9.0	220	24170	4.7	333	37546	9q21−22
cathepsin V (L2) O60911	17	96	11672	9.5	221	23999	8.6	334	37329	9q22.2
cathepsin S P25774	16	98	11830	9.3	217	23993	7.6	331	37479	1q21
cathepsin O P43234	23	84	9894	9.8	214	23460	5.8	321	35957	4q31−32
cathepsin F Q9UBX1	19	251	27846	9.4	214	23593	5.8	484	53365	11q13.1−3
cathepsin X (Z) Q9UBR2	23	38	4338	9.6	242	27149	5.5	303	33868	20q13
cathepsin B P07858	17	62	7245	9.6	260	28663	5.2	339	37807	8p22
cathepsin C P53634	24	206	23534	8.7	233	26032	5.6	463	51841	11q14.1−3
cathepsin K (O2) P43235	15	99	11833	6.3	215	23495	8.9	329	36966	1q21
cathepsin H P09668	22	75	9104	9.8	220	24187	5.7	335	37403	15q24−25
cathepsin W P56202	21	106	12060	4.8	249	28024	8.9	376	42099	11q13.1−3
Dust Mite										
D. pteronyssinus (Der p1) P08176	18	80	9273	5.4	222	25020	5.6	320	36104	
D. farinae (Der f1) P16311	18	80	9340	5.2	223	25192	5.8	321	36435	
Plasmodium										
P. falciparum (falcipain-1) P25805	17	315	38118	9.2	237	26864	5.3	569	66880	
falcipain-2 Q9NBD4	21	239	28490	9.5	224	24999	4.9	484	55992	
falcipain-3 Q9NBA7	17	247	28936	9.5	224	25108	4.7	488	56105	
P. cynomolgi Q25660		1−99			233	25931	5.2	332	38027	
P. berghei Q25644		1−98			231	26392	5.0	329	38166	
P. vinckei P46102		1−262			244	27561	4.8	506	58255	
P. vivax P42666		1−338			245	27254	5.1	583	65721	
Trypanosoma										
T. cruzi (cruzipain) P25779	18	104	11541	7.2	345 (129)	36459 (13716)	5.1	467	49836	
T. brucei (brucipain) P14658	20	105	11945	9.8	325 (108)	34212 (11131)	4.5	450	48451	
T. rhodesiense (rhodesain) Q95PM0	20	105	11945	9.8	325 (109)	34193 (11244)	4.4	450	48432	
T. congolense (congopain) Q26895	20	105	11952	9.5	319 (104)	33947 (10859)	5.2	444	48099	
cruzipain-B O61066	18	71	8058	10.2	244	26584	5.0	333	36773	
Leishmania										
L. major P90628	21	104	11856	9.5	318 (101)	34219 (10905)	6.4	443	48057	
L. mexicana P36400	27	98	11246	9.1	318 (99)	33958 (10538)	5.0	443	47728	
L. pifanoi Q05094	19	105	11886	9.3	320 (99)	34158 (10538)	4.9	444	47857	
L. donovani O18698	21	104	11854	9.7	318 (99)	34106 (10678)	5.4	443	47914	
L. major-B P90627	20	77	8250	6.1	256	28498	4.9	340	37246	
L. chagasi-B Q9GQN7	20	77	8355	5.0	243	26597	5.1	340	36977	
L. mexicana-B Q25319	20	76	8168	5.7	244	26924	5.2	340	37200	
Giardia										
G. lamblia-B1 Q9GP25	17	61	97085	10.0	225	24773	4.5	303	33550	
G. lamblia-B2 Q9GP24	20	54	6061	10.5	226	24976	4.7	300	33002	
G. lamblia-B3 Q9GP23	19	54	6061	10.5	226	24726	4.5	299	32674	
G. muris O15555	13	63	7291	6.7	225	25210	4.8	301	33706	
Entamoeba										
Entamoeba histolytica Q01957	13	80	9321	9.5	222	24169	5.1	315	35056	
Schistosoma										
S. japonicum Q10834	16	99	12220	5.9	216	24509	8.9	331	38460	
S. japonicum-B P43157	17	72	8464	5.9	253	28409	8.2	342	38796	
S. japonicum-C O18533	22	199	23372	9.6	237	26680	5.9	458	52698	
S. mansoni Q26534		1−104			215	24113	5.0	319	36136	
S. mansoni-B P25792	17	71	8494	6.5	252	28295	8.5	340	38592	
S. mansoni-C Q26563	20	197	22491	9.3	237	26436	4.8	454	51281	
Fasciola										
F. hepatica-1 Q24940	17	90	10881	6.3	220	24319	6.9	326	36896	
F. hepatica−2 Q24941	17	90	10942	8.2	219	24411	4.9	326	37177	
F. gigantica Q9XYL8	17	90	10907	5.6	219	24097	5.1	326	36771	
Other										
Paragonimus westermani Q9U0C8		1−210			217	23913	5.4	427	48652	
Ascaris lumbricoides P90685	17	103	12169	9.8	278	31236	5.1	398	45210	
Ancylostoma caninum-B Q11006	13	76	8805	4.6	254	28740	8.9	343	38881	
Necator americanus Q9U3938	14	69	8111	8.9	256	29191	8.4	339	38802	
Trichuris suis O17431	16	67	7458	5.5	265	29611	8.9	348	38791	
Ostertagia ostertagi P25802	19	69	8046	4.0	253	28156	7.6	341	38439	
O. volvulus Q9BJM3	23	164	19318	9.1	214	23607	9.3	401	45511	
Haemonchus contortus-B P19092	18	68	7795	4.8	256	28723	8.3	342	38459	
Heterodera										
H. glycines O18455	16	139	16218	8.2	218	24082	5.6	374	42231	
H. glycines-S O18456	33	101	11322	10.2	219	24126	5.1	353	38768	

Table 1 (Continued)

proteases AC number (Swiss-Prot)	signal peptide AA	prodomain length AA	prodomain size Da	prodomain pI	mature domain length AA	mature domain size Da	mature domain pI	prepro-enzyme length AA	prepro-enzyme size Da	chromosomal position
				Other						
Toxocara canis Q26888	27	117	14465	5.2	216	24260	4.9	360	41523	
Brugia pahangi O17473	16	165	19323	5.0	214	23529	8.6	395	44549	
Aedes aegypti-B Q16920	19	67	7555	9.6	256	28465	6.5	342	37897	
Boophilus microplus Q9NHB5	18	97	11068	9.7	217	23464	4.7	332	36313	
Sarcophaga peregrina Q26636	17	104	12464	9.6	218	23636	4.6	339	37847	
D. discoideum−2 P04989	18	104	12275	6.8	254	27455	5.6	376	41851	
				Theileria						
T. annulata P25781		1−213			214	233110	7.0	441	49653	
T. parva P22497		1−228			211	23406	7.0	439	50179	

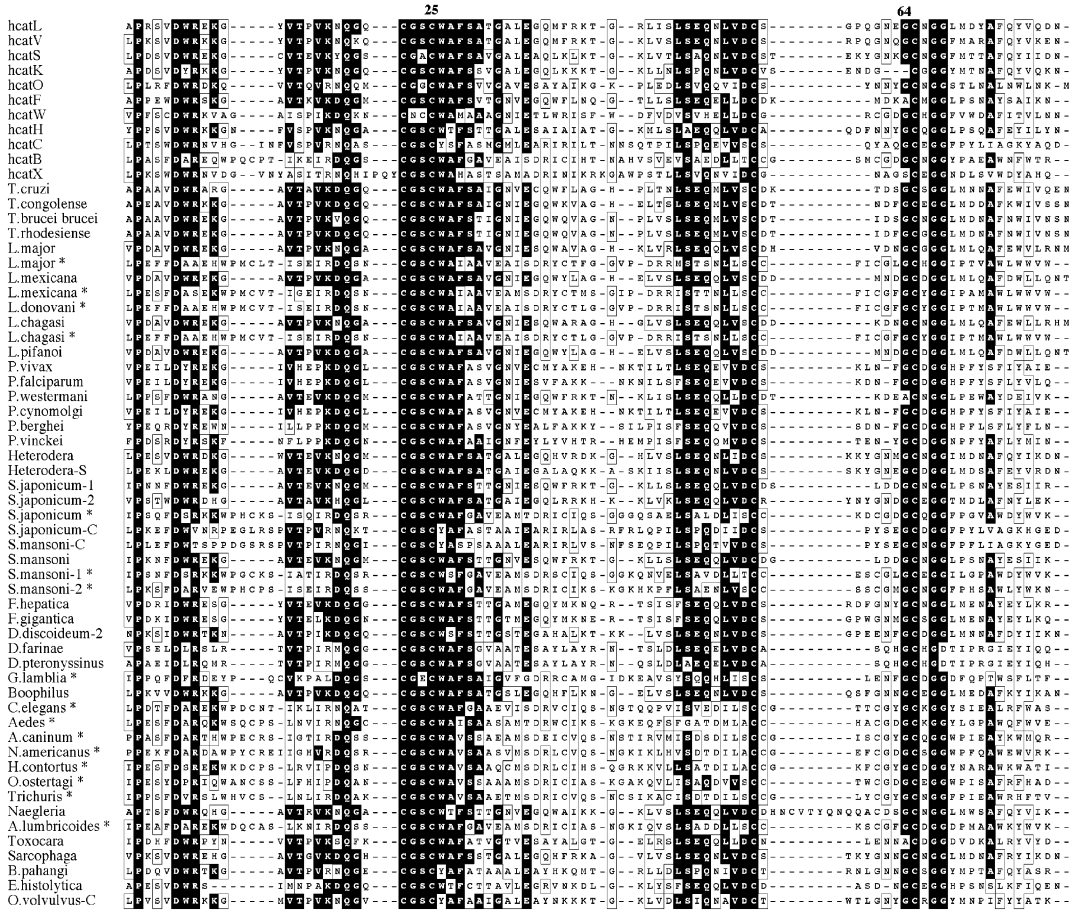
[a] Prediction of signal peptide and propeptide cleavage sites were performed by SignalP program from SWISS−PROT. C-terminal extension length and size (Da) for parasitic cysteine protease are indicated in parentheses.

cathepsin H appears to concentrate in the early endosomes of a macrophage cell line whereas the majority of cathepsin S is found in the late endosomes.[22] However, not all papain-like cathepsins are lysosomal enzymes and some use mannose 6-phosphate independent pathways for their transport into lysosomes or equivalent compartments. Human cathepsin W appears to be retained in the endoplasmic reticulum (ER) although it possesses one putative N-glycosylation site in its catalytic domain.[23,24] None of the known ER retention signals are present in the cathepsin W polypeptide suggesting the utilization of a yet unknown signal. On the other hand, some cysteine proteases with putative N-glycosylation sites may use a mannose 6-phosphate independent pathway for lysosomal targeting. For example, it has been suggested that cruzipain, a cathepsin L-like protease from *Trypanosoma cruzi*, relies on a nine amino acid sequence (FKQKHGRVY) of the propart for the delivery into the lysosomal compartment.[25] Similar propeptide motifs have been identified in cysteine proteases expressed in *Leishmania*.[26] Mannose-6 phosphate independent trafficking pathways also have been proposed for cathepsins B and H.[27,28]

With the exception of cathepsin S,[29,30] human cathepsins have an acidic pH optimum which allows full activity within the lysosomal compartment.[31] Most mammalian cathepsins are rapidly inactivated at neutral pH, an adaptive change that might represent a protective mechanism against accidental leakage from the lysosomes into the cytosol.[32] In contrast, many parasitic cysteine proteases are most active at neutral or slightly alkaline pH.[26,33,34] Neutral or alkaline pH optima are in accordance with the extracellular activity observed for these proteases. Extracellular functions of parasitic cysteine proteases in nutrition, tissue and cell invasion, ex/encystment, hatching and immunoevasion have been recently discussed in detail by Sajid and McKerrow (2002),[26] and the interested reader is referred to this review.

Human cathepsins are expressed either ubiquitously or tissue and cell specifically. Cathepsins B, L, H, C, X, F, and O are found in many organs and may fulfill housekeeping functions.[31,35−40] Whereas cathepsins L, S, K, V, F, and O are endopeptidases,[39] cathepsin X is a carboxypeptidase.[41] Cathepsins H, B, and C exhibit endo- as well as exopeptidase activities.[31,42,43] In addition to their endopeptidase activities, cathepsin H acts as an aminopeptidase,[44] cathepsin B acts as a dipeptidyl carboxy peptidase,[45,46] and cathepsin C, also known as dipeptidyl peptidase I, cleaves dipeptides from the N-terminus of peptides and protein substrates. The combined endo- and exopeptidase activities of lysosomal cathepsins warrant an efficient breakdown of protein substrates in lysosomes. The "mixed" activities of all three enzymes have been explained by their 3-D structures. As mentioned, cathepsin B possesses an occluding loop with two histidine residues which anchor the C-terminal carboxyl group of a protein or peptide substrate.[14,47] The endopeptidase activity of cathepsin B as well as its inhibition by cystatins and its propeptide depends on the flexibility of the occluding loop.[48,49] Removal of the occluding loop leads to a significant increase in the endopeptidase activity of cathepsin B.[50] In the cathepsin H structure, an octapeptide mini-chain attached via a disulfide bond to the body of the enzyme fills the region that in related enzymes comprises the nonprimed substrate-binding sites from S2 backward. Guncar et al.[51] suggested that the negatively charged carboxyl group of the C-terminus of the mini-chain acts as an anchor for the positively charged N-terminal amino group of a substrate and thus allows the aminopeptidase activity. The dipeptidyl aminopeptidase activity of cathepsin C is caused by the formation of a tetramer and a so-called exclusion domain which excludes the approach of a polypeptide chain apart from its termini. By anchoring the N-terminal amino group of the substrate to the carboxylic group of Asp^1 of the protease amino acid sequence the aminopeptidase activity can be explained.[52] The major physiological function of cathepsin C appears to be the activation of granzyme zymogens which requires the proteolytic removal of an N-terminal activation dipeptide.[53]

Recent studies have demonstrated that cathepsin activities and their stabilities can be regulated by glycosaminoglycans.[54] Almeida et al.[55] demonstrated that heparin and heparan sulfate specifically bind to cathepsin B and potentiate its endopeptidase activity as well as stabilize the protease activity at neutral pH by reducing the loss of cathepsin B α-helix content. We have recently shown that the collagenolytic activity of cathepsin K requires the formation of a complex between the protease and chondroitin sulfate. In the absence of chondroitin sulfate, monomeric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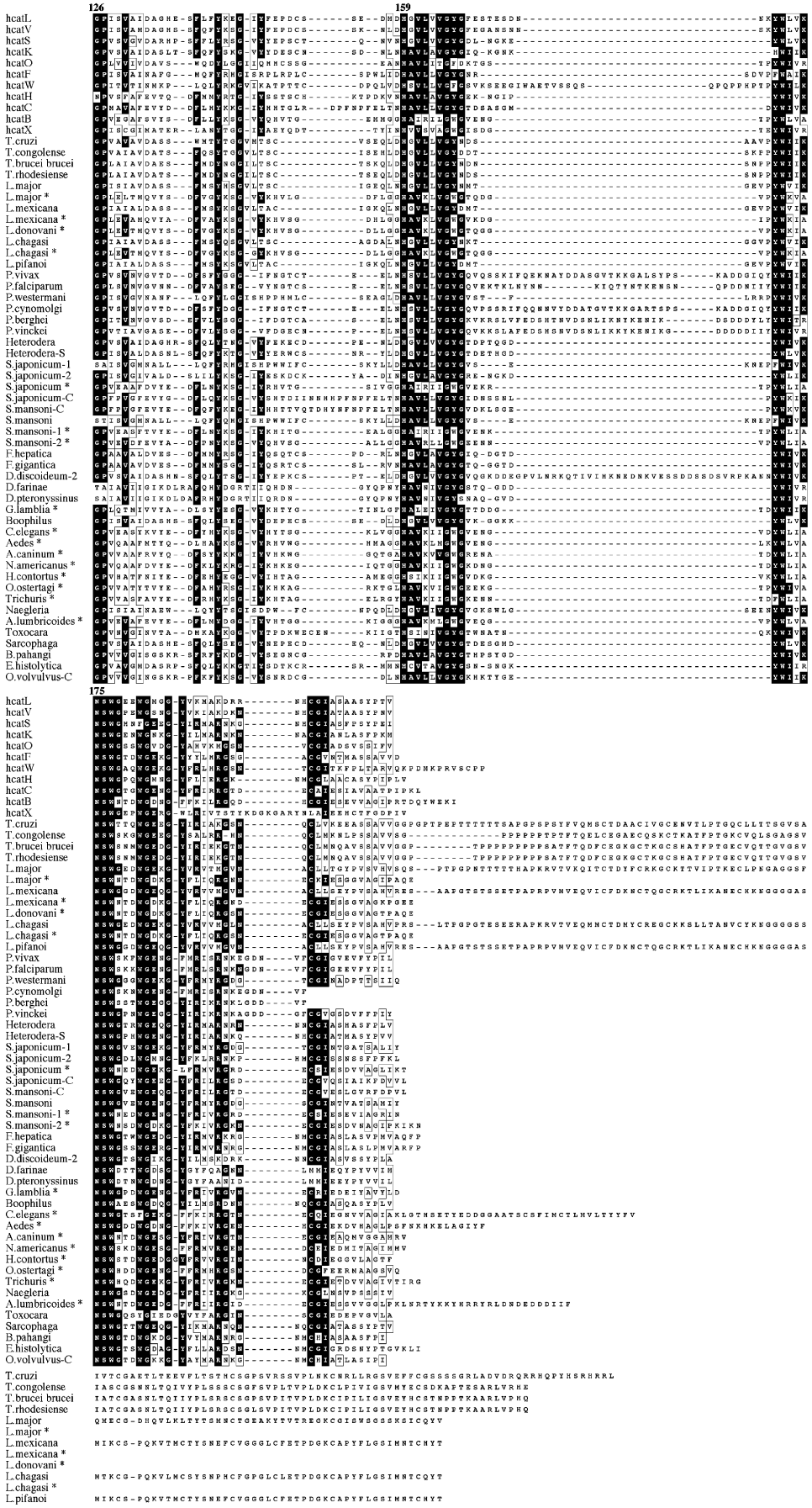


Figure 1. Amino acid sequence alignment of human lysosomal cathepsins and related parasite cysteine proteases. The sequences were taken from the SWISS−PROT databases and their alignment was performed by CLUSTAL_W program under MacVector software. Cathepsin B-like proteases are marked with asterisks.

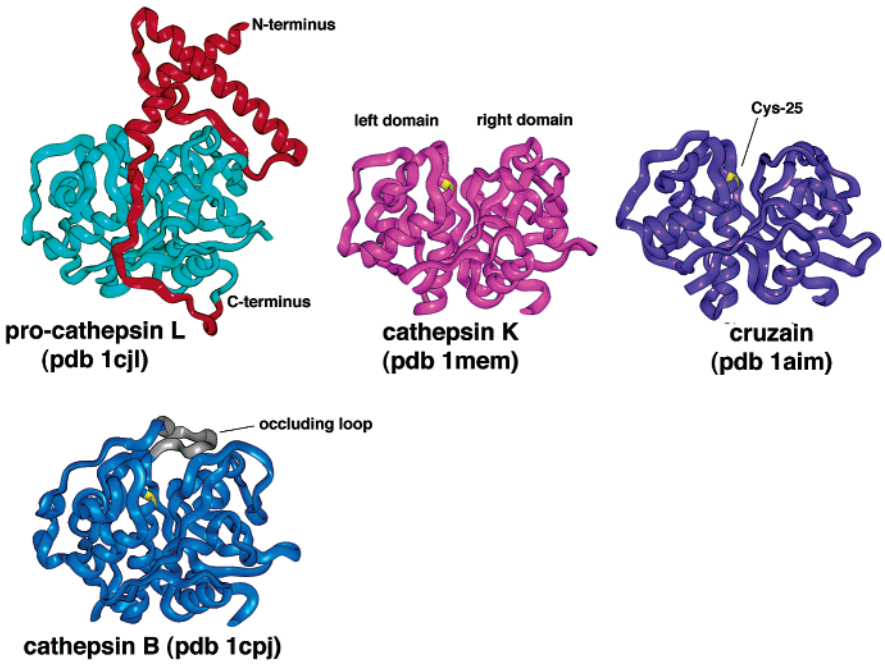


Figure 2. 3-D structures of human and trypanosomal cysteine proteases. Cysteine 25 is colored in yellow in all 3-D structures (ribbon representation). The cathepsin L propeptide is indicated in red and the occluding loop of cathepsin B in gray.

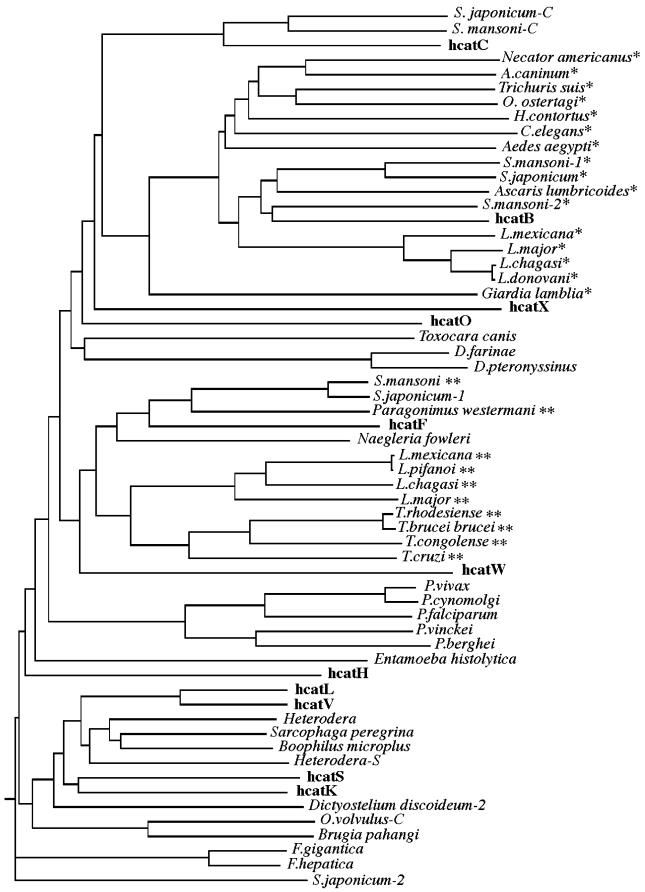


Figure 3. Phylogenetic tree of human and parasite papain-like cysteine proteases. Human cathepsins are highlighted in bold. Cathepsin B-like proteases are indicated with one asterisk (*), and cathepsin F-like enzymes with two asterisks (**).

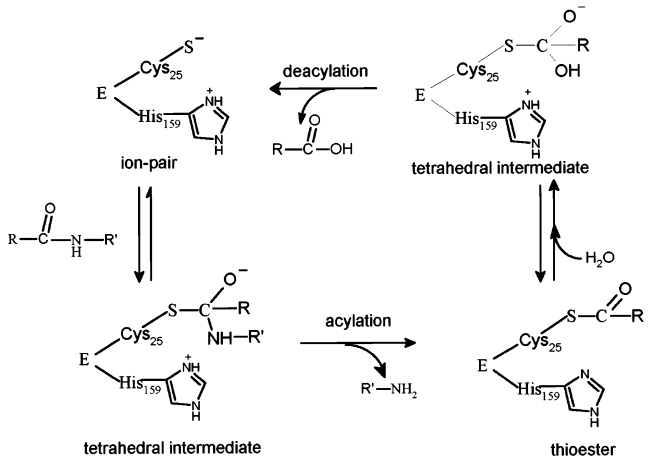


Figure 4. Mechanism of substrate hydrolysis by papain-like cysteine proteases.

cathepsin K does not degrade triple helical collagen but, on the other hand, retains its full activity toward gelatin and synthetic substrates.[56,57] A third example of glycosaminoglycan−cysteine protease interactions is represented by the finding that heparan sulfate enhances the kinin-degrading activity of cruzipain, the major cysteine protease activity in *T. cruzi*. It also reduces the inhibitory potency of high molecular weight kininogens against the protease. Interestingly, it was shown that kinin release by living trypomastigotes increased up to 10-fold in the presence of heparan sulfate suggesting that the efficiency of *T. cruzi* to initiate kinin release is potently enhanced by the mutual interactions between cruzipain, high molecular kiningens, and heparan sulfate proteoglycans.[58]

It should be noted that data about tissue distribution were frequently derived from Northern blot analyses of whole organs. This may hide a more cell type specific expression pattern of apparently ubiquitously expressed cathepsins. For example, the distribution of cathepsin F appears to be ubiquitous

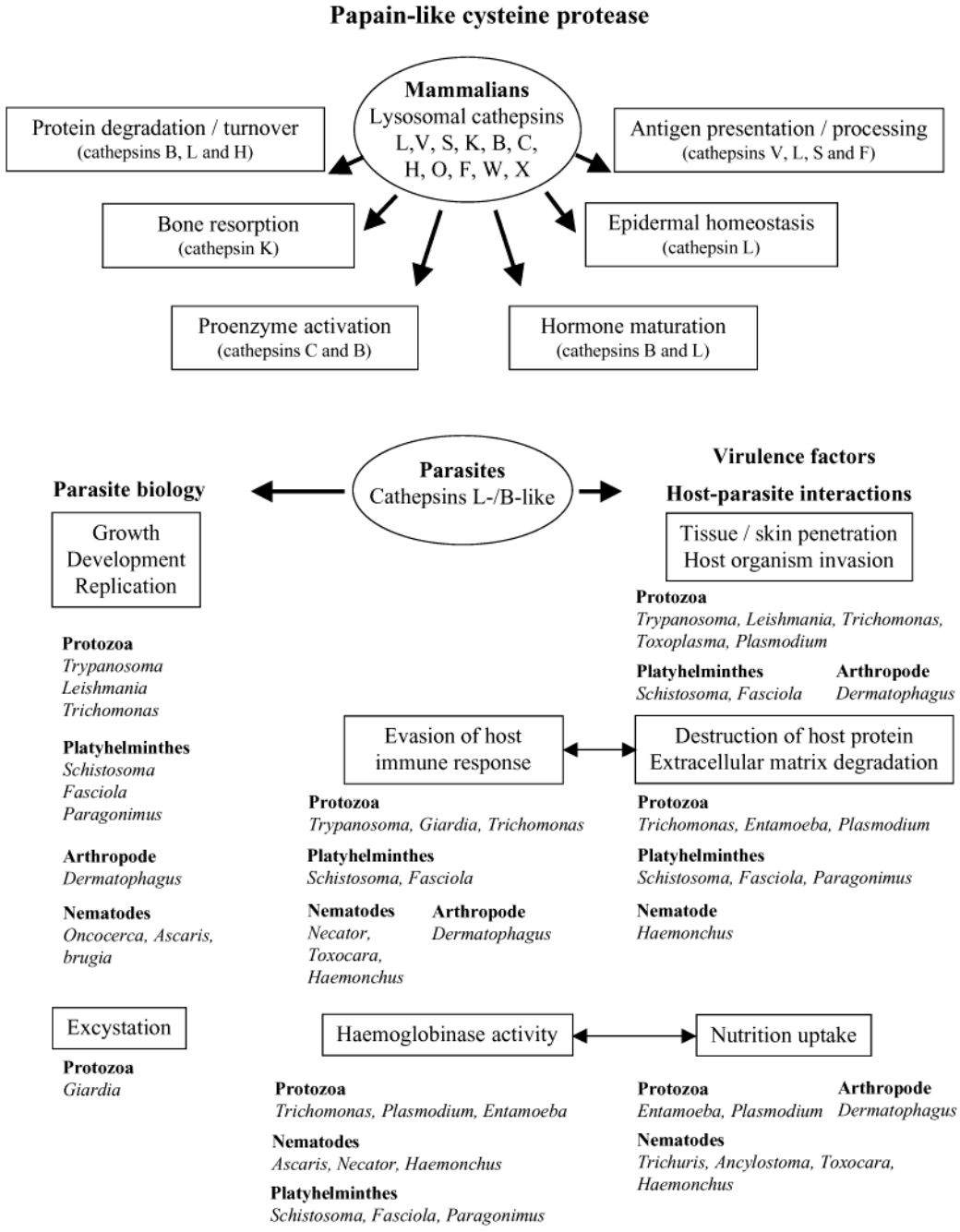


Figure 5. Physiological roles of human and parasitic papain-like cysteine proteases.

in Northern blot analysis,[40] whereas preliminary immunohistochemical analysis of cathepsin F expression revealed a preferential expression of the enzyme in macrophages and smooth muscle cells (unpublished observation, DB). Due to the widespread distribution of these cells in various tissues, the apparent nonspecific expression of the protease might be explained. In addition to cathepsin F, cathepsins S, K, V, and W have a highly selective tissue expression.[23,30,35,59−62] Cathepsin S is selectively expressed in lymphatic tissues and more specifically in antigen-presenting cells such as dendritic cells and macrophages as well as in smooth muscle cells[29,63,64] and plays a critical role in antigen presentation[65,66] (See section 3.1.5.). Closely related cathepsin K is highly and specifically expressed in osteoclasts and multinucleated giant cells responsible for bone and foreign body material degradation[60,67−69] (See section 3.1.1.). However, the expression of cathepsin K is not limited to osteoclasts and osteoclast-like cells. High expression levels have been identified in synovial fibroblasts which are involved in the pathological erosion of articular cartilage[70−72] (see section 3.1.2.) and in various epithelial cells.[73−75] A specific function of cathepsin K in these cells is unknown and the enzyme may fulfill house-keeping functions. Cathepsin V which shares 80% amino acid sequence identity with cathepsin L is selectively expressed in thymus, testis, and corneal epithelial cells.[59,62,76] Its selective expression in thymic cortical epithelial cells and its ability to degrade the invariant chain of MHC class II complexes suggest a pivotal role of cathepsin

V in the positive selection process of T cells (unpublished observation, DB). Cathepsin W (also known under the synonym lymphopain) is selectively expressed in cytotoxic natural killer cells.[23,24,61] To date, neither a function nor a catalytic activity has been described for this protease. Nevertheless, the selective expression in natural killer cells implies a specific role in the pathway of the cytotoxic killing of abnormal cells.

Figure 5 summarizes the physiological functions of mammalian and parasite papain-like cysteine proteases. Pathophysiological implications of cysteine proteases will be discussed in the following chapter.

3. Pathophysiological Implications of Papain-Like Cysteine Protease Activities

To date, two human cathepsin deficiencies have been described: (i) pycnodysostosis, a skeletal bone dysplasia caused by cathepsin K deficiency,[77] and (ii) Papillon-Lefevre syndrome, a periodontopathia caused by cathepsin C deficiency.[78] Both diseases are rare and might be cured by bone marrow transplantation or gene therapy. The major role of papain-like cysteine proteases in human pathology, however, is not related to enzyme deficiencies but to the overexpression of these proteases or their "out of place" activities. Papain-like cysteine proteases have been increasingly recognized as critical enzyme activities in degenerative, invasive, and immune system related disorders as well as in various parasitic infections. In this chapter, we want to summarize the role of cysteine proteases in diseases that have a large impact on society in the industrial as well as developing world. The understanding of the functions of these proteases in physiology and pathology will allow the design of selective therapeutic agents. The interested reader is also referred to a previous review focusing on the emerging roles of cysteine proteases in human biology.[79] Major advances in cysteine protease targeted drug discovery have been accomplished for diseases with excessive bone resorption such as osteoporosis[80] and parasite induced diseases such as Chagas' disease.[81] Recent developments in cysteine protease inhibitor design will be addressed in chapter 4.

3.1. Systemic Human Diseases

3.1.1. Osteoporosis

Osteoporosis is a disease characterized by an imbalance between bone resorption and formation. Excessive bone resorption causes changes in the microstructure of the bone matrix which makes bones prone to fracture. In 1990, an estimated 1.7 million osteoporosis-related hip fractures occurred worldwide from which approximately 25% had a lethal outcome. Currently, 44 million people or 55% of the US population aged 50 or older have osteoporosis or are at risk to develop the condition during their lifespan (http://nof.org/advocacy/prevalence). Direct and indirect costs of osteoporosis are staggering and estimated to be $18 billion annually and increasing every year. Current therapies are mostly directed to decrease the rate of bone resorption. Antiresorptive therapies and compounds include the estrogen replacement therapy, selective estrogen receptor modulators, calcitonin, vitamin D, calcium supplements, PTH and PTH analogues, and bisphosphonates (summarized in ref 82). All therapies show efficacy but reveal various problems such as increased cancer rates in the estrogen-replacement therapy[83] or upper gastrointestinal symptoms and problems in patient compliance in the case of bisphosphonates.[84] Thus, other nonhormonal and more specific therapies are needed. In the following, we briefly discuss cathepsin K as a novel anti-osteoporotic drug target.

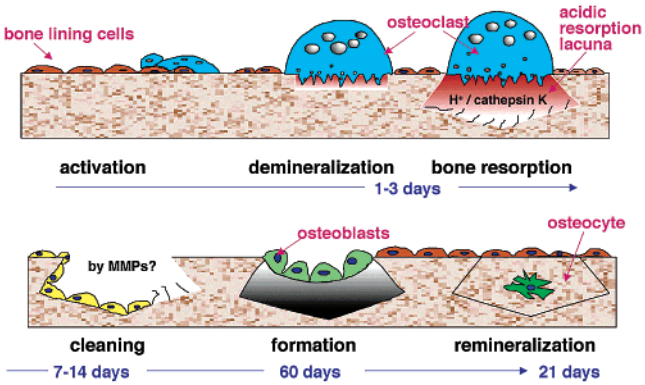


Figure 6. Bone remodeling cycle.

Bone tissue is renewed every 5 to 7 years throughout the skeleton by a process called bone remodeling. Bone degrading multinucleated osteoclasts formed from mononuclear precursor cells secrete protons and proteolytic enzymes which first demineralize the bone matrix and then degrade its collagenous organic component. After approximately 3 days, osteoclasts leave behind an excavation pit which is refilled with fresh bone matrix by osteoblasts which usually takes 2 months for completion[85] (Figure 6). The major proteolytic activity of osteoclasts is cathepsin K which represents 98% of the total cysteine protease activity.[60] The physiologically relevant substrate of osteoclast-expressed cathepsin K is type I collagen which constitutes 95% of the organic bone matrix.[67] Cathepsin K is the only known mammalian collagenase activity capable to cleave inside triple helical collagens at multiple sites and to completely hydrolyze collagen fibrils into small peptides.[86,87] This activity is only comparable to that of bacterial collagenases.[88] The collagenolytically active form of cathepsin K is an oligomeric complex of cathepsin K and chondroitin sulfate molecules.[57] Cathepsin K also cleaves in the N- and C-telopeptide regions of collagen and releases cross-linked N- and C-telopeptides. These peptides can be determined in urine and serum by immunoassays[89,90] and serve as useful diagnostic markers of osteoclastic bone resorption in vivo. The collagen cleaving activities of other cysteine proteases such as cathepsins L and B are restricted to these sites although their activities are less efficient when compared to cathepsin K. Collagenases of the matrix-metallo proteinase family cleave selectively at one site in the triple helix to generate one-quarter and three-quarter fragments. Cathepsin K also efficiently degrades osteonectin.[91]

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, pycnodysostosis.[77] Osteoclasts from pycnodysostosis patients show an intracellular accumulation of undigested collagen fibrils[92] and patient carriers of the enzyme deficiency are characterized by an osteopetrotic phenotype. A comparable bone phenotype also was demonstrated in cathepsin K-deficient mice.[93] On the other hand, overexpression of cathepsin K in osteoclasts accelerated the turnover of metaphysical trabecular bone in mice.[94]

The critical role of cathepsin K in bone resorption led to the belief that specific cathepsin K inhibitors will be beneficial in the treatment of osteoporosis. Selective cathepsin K inhibitors have been tested in cell-based assays of bone resorption[95] as well as in rodent and nonhuman primate models of osteoporosis.[96,97] A small molecule inhibitor with a K_i of 0.16 nM significantly reduced serum markers of bone resorption in an estrogen-deficient monkey model of osteoporosis (see Table 2, example 27). N- and C-terminal telopeptides of type I collagen were reduced by 61 and 67%, respectively.[96] In contrast, selective cathepsin L inhibitors did not inhibit osteoclast-mediated bone resorption in vitro.[98] The design of specific cathepsin K inhibitors has been recently reviewed by Yamashita et al.[80,99]

3.1.2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a highly inflammatory joint disorder that affects 1−2% of the U.S. population. Direct and indirect costs are estimated to be over $14 billion annually. Similar to osteoporosis, three times more women than men are affected by the disease. Besides its inflammatory component, RA is characterized by a progressive articular cartilage and subchondral bone destruction that eventually leads to loss of joint function.

In both disease-defining components of RA, i.e., inflammation and cartilage and bone destruction, papain-like cysteine proteases are involved. Implications of cathepsins in inflammation and their role in antigen presentation will be discussed further below.

Cartilage consists of two major constituents: type II collagen forming a 3-D network of fibrils which gives the tissue its tensile strength and hydrophilic aggrecan aggregates which are responsible for the compressive stiffness of cartilage.[100] Excessive proteolytic degradation of either one or both components results in the destruction of articular cartilage.[101] Cartilage is attacked from three sites in RA: (i) from the smooth surface of articular cartilage by a pannus formed from synovial fibroblasts, macrophages, and infiltrated leukocytes, (ii) from activated chondrocytes within the cartilage, and (iii) from osteoclasts, osteoclast precursor cells and invading synoviocytes from the subchondral bone side (Figure 7). Metalloproteases such as collagenases and aggrecanases have been implicated in the destruction of the cartilage matrix. Collagenases of the MMP family (e.g., MMP1, MMP13) are upregulated by RA-inflammatory cytokines such as IL-1 and TNF-α and they

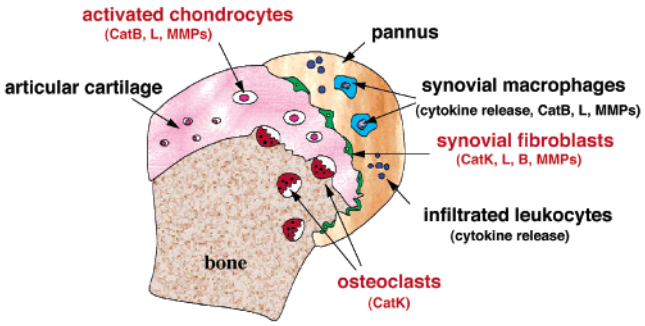


Figure 7. Tridirectional erosion of cartilage in arthritic joints and proteases implicated in cartilage destruction (CTSK, cathepsin K; CTSL, cathepsin L; CTSB, cathepsin B; MMP, matrix metalloproteaese).

have until recently been regarded as the only type II collagen degrading proteases.[102−104] Furthermore, aggrecanase, a member of the ADAMS-family, has been demonstrated to cleave selectively in the interglobular domain (IGD) of aggrecan.[105,106,107] However, increasing evidence suggests a critical role of papain-like cathepsins in the pathogenesis of RA as well. Cysteine protease specific inhibitors such as peptidyl fluoromethyl ketones or peptidyl vinyl sulfones have been show to reduce cartilage erosion and inflammation in adjuvant induced arthritis.[108−110] Mu-Leu-homoPhe-vinyl sulfone (also known as LHVS; see Table 2, example 38) reduced inflammation by approximately 40% and joint destruction by 66% when administered in food at 2.2 mg/kg/day for 4 weeks.[110] The peptidyl vinyl sulfone inhibitor LHVS is an extremely potent cathepsin S inhibitor but also shows potency to other cathepsins including cathepsin K and parasitic cysteine proteases.[111] Since it is generally accepted that inflammatory processes precede the destruction of cartilage and bone in RA, it is not clear whether the primary effect of LHVS is the reduction of inflammation or a simultaneous and direct effect on cartilage and bone degrading cysteine protease activities.

Which cathepsins are expressed in RA joints? Cathepsins L, B, and K have been identified in synovial fibroblasts,[72,112−114] and it has been demonstrated that they are inducible by IL-1, interferon γ, TNF-α, and FGF.[71,115] Macrophage-like type A synoviocytes express cathepsin S and at low levels cathepsin K.[71] The major protease activity in osteoclasts is cathepsin K,[35,60] whereas cathepsins B and L are the predominant cysteine proteases in chondrocytes.[116] Interestingly, Konttinen et al. observed recently the expression of cathepsin K in chondrocytes of advanced osteoarthritic cartilage.[117] The expression of cathepsin K in chondrocytes of RA-disease specimens has not yet been demonstrated. All cathepsins present in inflamed joints are capable to hydrolyze aggregan aggregates.[114,118,119] Of particular interest is that cathepsin S can degrade aggregan also efficiently at neutral pH,[114] whereas the activity of the other cysteine proteases is restricted to lysosomal acidic pH values. However, it should be noted that (i) extracellular matrix components such as glycosaminoglycans can stabilize otherwise alkaline pH labile cysteine proteases such as cathepsin B at alkaline pH[55] and (ii) that pH values

as low as pH 5 have been reported for diseased cartilage[117,120] which would provide an optimal extracellular environment for all cathepsin activities. In contrast to the hydrolysis of aggrecan, the degradation of type II collagen is restricted to activities of collagenolytic matrix metalloproteinases and cathepsin K.[87] Among all known human collagenolytic activities, cathepsin K activity alone is sufficient to degrade native type II collagen into small fragments. It is of particular interest, that the collagenase activity of cathepsin K requires cartilage resident glycosaminoglycans.[56,57] The potent collagenase activity of cathepsin K and its expression in cartilage degrading cells at the invasion front of the pannus suggest a critical role of cathepsin K in the degenerative pathology of RA.

A specific feature of RA is juxtaarticular osteoporosis due to subchondral bone resorption. There is clear evidence that osteoclasts and their mononuclear precursor cells are responsible for this process.[121] TRANCE/RANKL-deficient mice which are unable to form osteoclasts revealed decreased subchondral bone resorption in an RA mouse model.[122] Interestingly, cartilage erosion was not significantly decreased in these mice suggesting that the destruction of cartilage is not mediated by osteoclast-like cells. Cathepsin K has been demonstrated to be the major bone degrading activity in osteoclasts (see section 3.1.1.). Malformation of osteoclasts would affect the activity of cathepsin K in these cells. On the contrary, cartilage-degrading cells within the pannus were not affected by RANKL deficiency and thus should not affect the expression of cathepsin K in synovial fibroblast- and macrophage-like cells. It remains to be seen whether cathepsin K deficiency in mice will have a bone as well as cartilage preserving effect in a RA disease model. Besides osteoporosis, rheumatoid arthritis may represent the most promising clinical indication for the therapeutic use of selective cathepsin K inhibitors.

3.1.3. Atherosclerosis

Atherosclerosis is the major cause of death in the Western world. Approximately 50% of the mortality of the U.S. population is attributed to atherosclerosis-related disorders. Atherosclerosis is characterized by a patchy thickening of arteries causing a reduction or obstruction of blood flow that eventually results in myocardial or cerebral infarctions or aortic aneurysms. A typical early sign of atherosclerosis is the formation of fatty streaks which consist of lipid-laden macrophages accumulating in the subendothelial layer of the intima. Later, fatty streaks develop into fibrous plaques consisting of intimal smooth muscle cells, connective tissue, and lipids. Cathepsins S and K have been localized in macrophages and smooth muscle cells in atheromas.[64] Both enzymes have a strong elastin-degrading activity[67,123] suggesting a role in the progressive erosion of the elastic arterial walls during atherosclerosis. A selective inhibitor of cathepsin S blocked approximately 80% of the elastolytic activity of IL-1β or IFN-γ activated primary cultures of smooth muscle cells.[64] Shi et al.[124] reported that the expression of cystatin C, a potent endogenous cathepsin inhibitor, is dramatically reduced in both atherosclerotic and aneurysmal aortic lesions. Furthermore, increased abdominal aortic diameter among 122 patients screened by ultrasonography correlated inversely with serum cystatin C levels. These findings suggest an important role of an imbalance between cathepsin activities and their endogenous inhibitors in the pathogenesis of atherosclerosis. Besides cathepsins S and K, a third papain-like cysteine protease, cathepsin F, may play a critical role in the disease progression. During atherogenesis, low-density lipoprotein (LDL) particles become modified and fuse to form lipid droplets in the arterial intima. Proteolysis is one possible mechanism of droplet formation, since the lipid droplets contain degraded apolipoprotein apoB-100, and it has been shown that several neutral proteases can induce LDL fusion in vitro. We found cathepsin F within the macrophage-rich areas of the human coronary atherosclerotic intima and demonstrated the specific and efficient proteolytic degradation of apoB-100 by cathepsin F. The degradation of apoB-100 triggered the aggregation and fusion of LDL particles, which increased both the binding strength and accumulation of LDL to human arterial proteoglycans. This may indicate an important contribution of cathepsin F to the progressive accumulation of LDL-derived lipid droplets during atherogenesis. The specific inhibition of cathepsin F might be beneficial for the treatment of atherosclerotic diseases (Öörni and Brömme, unpublished data).

3.1.4. Cancer

Tumor progression and metastasis require local proteolysis for the spatial expansion of tumors, for the generation of tumor supporting blood vessels (angiogenesis) and for the migration of transformed cells in and out of the vascular system (metastasis). Proteolytic activities from all major protease classes including papain-like cysteine proteases have been implicated and it is highly likely that these activities are organized in activation cascades. For example, procathepsin B can be activated by the aspartic protease, cathepsin D, by tissue and urokinase plasminogen activators (tPA and uPA), or by the serine protease, cathepsin G.[125,126] Active cathepsin B then can convert more uPA zymogen and plasminogen into their proteolytically active forms which have been demonstrated to have matrix metalloproteinase activating properties.[127,128] The same cascade of activation might also be true for cathepsin L.[129]

Proteases required for tumor invasion and metastasis can be expressed by tumor cells as well as surrounding stromal cells. For example, invading tumor cells may activate protease expression in stromal fibroblasts which assist in the degradation of the extracellular matrix. It has been shown that fibroblasts neighboring tumor cells have elevated levels of gelatinase B (MMP9).[130] We have shown that in breast tumor samples, cathepsin K is expressed in fibroblast-like cells (unpublished observation, DB). Considering the potent collagenase activity of cathepsin K it is conceivable that the enzyme contributes to the tumor expansion.

Increased expression levels of cathepsin B have been observed at the invasive edge of various tumors including bladder, colon, and prostate carcinomas.[131−134] Elevated levels of cathepsin L have been reported for kidney and testicular tumors, nonsmall cell carcinomas of the lung and in most cancers of the breast, ovary, colon, adrenal, bladder, prostate, and thyroid.[135] A specific role of cathepsin B in extracellular matrix degradation is supported by the findings that the protease reveals intracellular redistribution of its expression pattern in tumor cells when compared with normal cells. Campo et al.[136] observed that in colon carcinomas cathepsin B was redistributed from the apical region to the basal plasma membrane suggesting a role in basal membrane degradation. Moreover, it was demonstrated that cathepsin B can specifically bind to external cell surface proteins which increases the pH stability of the protease[137−139] and that cathepsin B co-localizes with the uPA receptor on cell surfaces.[140] Inhibition of cell-surface cathepsin B can prevent the activation of uPA, a well-known prognostic marker in cancer. Besides the expression of cathepsin B, also cathepsins L, H, and S have been correlated with tumor progression.[141] For example, high serum levels of cathepsin B and H in colorectal cancer patients correlated with shorter survival. The role of papain-like cathepsins is also supported by the finding that imbalances of protease and endogenous inhibitor expression tend to be higher in patients with malignant than with benign tumors.[141] Increased cathepsin L expression has been reported in invasive types of meningiomas.[142] However, it should be noted that data identifying cathepsins as diagnostic markers remain sketchy and in part contradicting. More studies are necessary to validate the relevance of those observations. The interested reader is referred to detailed reviews about the potential role of cathepsins in cancer.[141,143−145]

3.1.5. Inflammation and Immune System-Related Diseases

Although inflammation and immune responses are protective mechanisms of the organism to fight tissue injuries and infections, they are frequently connected to acute and chronic ailments such as rheumatoid arthritis, inflammatory bowel disease, myasthenia gravis, or asthma. A common feature of these diseases is the presentation of exogenous antigens or autoantigens by the major histocompatibility complex of class II (MHC class II) which leads to appropriate immune responses. To launch a specific T-cell mediated immune response, T cells must recognize an antigen which has been processed and presented by antigen-presenting cells (APCs). T-cell activation by peptides bound to MHC class II molecules on APC is preceded by the endocytic uptake and processing of antigen by endosomal-lysosomal proteases. Two proteolytic events are required for MHC class II-mediated antigen presentation: (i) degradation of the antigen to antigenic peptides capable to bind in the binding grove of MHC class II complexes and (ii) degradation of the invariant chain (Ii) which blocks the binding of the antigenic peptide to the MHC grove (reviewed in refs 146−148) (Figure 8). It is generally

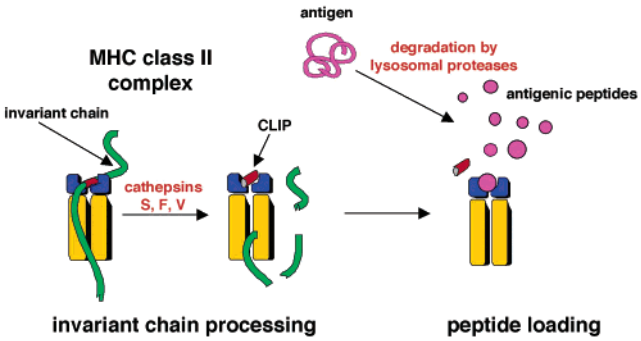


Figure 8. Role of cathepsins in MHC class II presentation.

accepted that cysteine and aspartic proteases participate in both processes. Antigen processing is probably a multiprotease-catalyzed process, and it proved difficult to identify individual proteases essential for processing. Gene deletion experiments of ubiquitously expressed aspartic and cysteine proteases such as cathepsins D and B revealed that antigen processing in mutant mice was not impaired thus indicating that redundant protease activities can replace cathepsins D and B.[149] To date, the only antigen-processing specific protease described is the nonpapain-like cysteine protease, asparagine endopeptidase (AEP). AEP appears to be essential for the processing of the microbial antigen tetanus toxin.[150,151] Specific progress has been made to understand the role of cysteine protease of the papain family in the degradation of the Ii chain.[65,66,152] Ii is a 31-kDa chaperone molecule which binds to α,β MHC class II molecules and guides them into the endosomal compartment. The most intimate contact between MHC class II molecules and Ii is in an approximately 15 amino acid long peptide region, called CLIP. CLIP binds similarly to an antigenic peptide in the binding groove of the MHC complex and is replaced by an antigenic peptide during peptide loading. To remove Ii from the MHC class II molecule, Ii must be degraded.[153] It has been demonstrated by in vitro and in vivo experiments that papain-like cathepsins are essential for the last processing step of the Ii, which leads to the 3 kDa CLIP peptide from a 10 kDa intermediate precursor.[66,154] The first cysteine protease identified as a CLIP releasing proteolytic activity was cathepsin S.[66] Using the highly potent vinyl sulfone cathepsin inhibitor, LHVS, which is specific for cathepsin S at nanomolar concentration, it was demonstrated that invariant chain processing and peptide loading could be blocked in bone-marrow-derived antigen-presenting cells such as B-cells, and dendritic cells.[66] The characterization of cathepsin S deficient mice confirmed these findings in vivo.[154,155] Interestingly, cathepsin S deficiency significantly reduced the susceptibility of mice to collagen-induced arthritis suggesting that a selective inhibition of cathepsin S might be beneficial for the treatment of rheumatoid arthritis.[155]

A cathepsin S inhibitor of the aldehyde class (see example 11 from Table 2) has been recently shown to be efficacious in a murine model for Sjögren syndrome. The compound profoundly blocked lymphocyte infiltration into the salivary and lacrimal glands, stopped antibody production and decreased

the symptoms of the autoimmune disease. It was shown that the cathepsin S inhibitor markedly impaired the presentation of α-fodrin antigen, an organ-specific autoantigen in Sjögren syndrome.[156]

Experiments with cathepsin S-deficient mice also demonstrated that cathepsin S is not universally responsible for the generation of CLIP in all types of APC. For example, antigen presentation in macrophages was less affected in the absence of cathepsin S[155] indicating that either Ii-chain processing cathepsin activities are redundant in these APCs or that distinct APCs express cell/tissue-specific cathepsins. Indeed, Shi et al. could show that in cathepsin L and S-deficient macrophages, cathepsin F can efficiently process Ii.[157] Cathepsin F is a recently described member of the papain-family which appears to be preferentially expressed in macrophage-like cells.[38,40] On the other hand, Nakagawa demonstrated that cathepsin L-deficient mice exhibit an impairment of thymic antigen presentation resulting in low numbers of $CD4^+$ cells, and suggested that cathepsin L activity is responsible for the final degradation of Ii in cortical thymic epithelial cells.[158]

We have recently identified and partially characterized a novel human cysteine protease closely related to cathepsin L which is named cathepsin V. Cathepsin V shares 80% protein sequence identity with cathepsin L, but, in contrast to ubiquitously expressed cathepsin L, its expression is restricted to thymus, testis, and corneal epithelium.[59,62,76,159] We have localized cathepsin V in antigen presenting thymic cortical epithelial cells and have demonstrated the efficient processing of Ii by this cysteine protease (Telosa and Brömme, unpublished results). It is conceivable that in human thymus cathepsin V takes over the function described for cathepsin L in mouse thymus. It is of particular interest that cathepsin V is overexpressed in thymii of myasthenia gravis patients. Myasthenia gravis is an autoimmune disease of the neuromuscular system, and it can be hypothesized that the overexpression of cathepsin V facilitates autoimmune reactions.

3.1.6. Allergic Inflammation/Asthma

House dust mites, *Dermatophagoides pteronyssinus* and *D. farinae*, are the most important causative factors associated with allergic diseases and to the rising incidence of asthma. Some studies have indicated that two highly related cysteine proteases (Der p1 and Der f1) are major house dust mite allergens found in feces of the ectoparasites.[160] They are expressed in the alimentary tract where they participate in the digestion of food.[161] The immunodominant allergen Der p1 is able to cleave IgE receptor (CD23) on B-lymphocytes and CD25 receptors,[162] resulting in the specific enhancement of IgE production.[163] Molecular modeling of Der p1 on the basis of other papain-like structures[164] revealed potential epitopes for IgE and IgG binding.[165] The mite protease is able to disrupt tight junctions of human lung epithelium cells, thus increasing the transepithelial passage of itself and other antigens into blood vessels.[166] This phenomenon may be more prominent in the airways of asthmatic patients, since Der p1 inactivates the serine protease inhibitor α_1-antitrypsin, a major component in the protection against proteases in the human respiratory tract.[167]

3.2. Parasitic Infections

Parasitic diseases are distributed predominantly in endemic areas such as Africa, Asia, Central and South America and are relatively rare in industrialized countries. Increasing incidences of leishmaniasis, however, have been recently reported for Southern Europe.[168] Many human and domestic animal parasitic infections require transmission by local vectors involving sometimes an intermediate host, by skin infections or rarely by blood transfusions or organ transplants. According to the World Health Organization, 3.5 billion people or more than 50% of the world population suffer from some type of parasitic infection. Most of them result in chronic infection, causing severe morbidity in some cases. Despite various efforts to control the spread of major public health affecting parasitic diseases such as malaria, bilharzia, amebiasis, Chagas' disease, leishmaniasis or African sleeping sickness, the overall incidence of these diseases is increasing.[169−172] These infectious diseases have received only limited attention from pharmaceutical companies toward the development of new drugs[173−175] and vaccines.[176] Furthermore, most of the drugs currently in use were designed decades ago and exhibit decreasing efficacy due to increasing resistance of the causative parasites and show various serious side effects making them inappropriate for long-term treatment.[175,177,178] At present, there are no vaccines available for human and domestic animals parasitic infections.[179] In addition, the control of these diseases is hampered by the lack of understanding the mechanisms of action of most antiparasitic drugs and by the lack of mechanism-based novel drug targets within the parasites. Parasitic cysteine proteases may fill this void. In contrast to a simple digestive role, parasite derived cysteine proteases have been characterized to perform numerous indispensable roles in the biology of many species of parasites (e.g., in replication or growth, cell differentiation, signaling, host invasion).[180−185] Several of these proteases have been shown to be virulence factors by proteolytically degrading components of the host immune system including immunoglobulins and components of the complement system. They are able to degrade extracellular matrix proteins and enhance the processing of various zymogens such as procollagenases and proenzymes of the clotting system, or exhibit a kininogenase activity and release bradykinin.[186−190] Promising initial experiments using cysteine protease inhibitors have validated the suitability of these proteases as drug target candidates for antiparasitic chemotherapy.[26,180,187,191−193] This chapter will focus on major parasitic diseases and summarize the physiological functions of their papain-like cysteine proteases.

3.2.1. Malaria

Human malaria is caused by infection with one of four species of the protozoa *Plasmodium* (*Plasmo-*

dium falciparum, *P. vivax*, *P. malariae*, and *P. ovale*) and represents the most prevalent tropical disease (300−500 million people are infected worldwide per year; http://www.who.int/tdr/diseases/malaria/default.htm). Infections with *P. falciparum*, the most virulent human malaria parasite, cause more than one million deaths annually, especially in children under the age of five. The protozoa is transmitted from human to human by female *Anopheles* mosquitos. The basic elements of the life cycle of the parasite are the same for all four species. Gametocytes from the blood of an infected person reproduce sexually inside the mosquito and develop into infective sporozoites within one to two weeks. When the mosquito feeds on a human, it inoculates sporozoites, which quickly infect hepatocytes. After rupture of the cells where cysteine proteases are likely to be involved,[194] merozoites are released and invade erythrocytes which are eventually destroyed.[195] This erythrocytic life cycle is responsible for all clinical manifestations of malaria (fever, paroxysm, anemia, and jaundice). A separate cycle of development of the parasite (formation of gametocytes) occurs in the plasma maintaining the parasite's life cycle by infecting another mosquito.

Antimalarial drugs are chloroquine, quinine, mefloquine which increasingly generate resistance especially in *P. falciparum*.[196] Promising new drug targets are hemoglobin-degrading cysteine proteases in *P. falciparum*.[192] *P. falciparum* expresses three papain-like thiol proteases named falcipain-1, -2, and -3. In concert with two aspartic proteases named plasmepsins I and II,[197] falcipains play a critical role in hemoglobin hydrolysis to provide amino acids for the development of the parasite in erythrocytes.[195,198,199] Falcipain-2 and -3 (65% of amino acids identity) are predominantly expressed in the merozoite acidic food vacuole (analogous to lysosomes) where the hemoglobin hydrolysis occurs.[200,201] Falcipain-3 appears to be the more efficient hemoglobinase than falcipain-2. The function of falcipain-1 is the less understood due to its low abundance in trophozoites and its heterologous expression pattern.[202] The primary subsite specificities of falcipains are similar with a preference for P2 leucine residues over phenylalanine and valine.[201] All three enzymes possess unusually long prosequences (2−3 times longer than those of most mammalian and parasite cysteine proteases) which may act as membrane anchors.[201,203] For a deeper discussion of malarial cysteine proteases, the interested reader is referred to a recent review by Rosenthal et al.[204]

3.2.2. American Trypanosomiasis (Chagas' Disease)

Among the trypanosomatid flagellated protozoae, Chagas' disease causing *T. cruzi* is the most important species from a public health point of view. The parasite is found throughout much of Central and South America. Chagas' disease is the leading cause of cardiomyopathy in these countries and affects more than 16 million people (http://www.who.int/tdr/diseases/chagas/default.htm). Isolated cases have also been reported in the United States. In addition to the human host, *T. cruzi* is also found in various animals including domestic animals and rodents, but its transmission to humans via these species remains unclear. *T. cruzi* is transmitted to humans either by triatomine vectors (kissing bug) or infected blood products and is found as an intracellular form, the amastigote, and as a trypomastigote form in the human blood. In the vector, noninfective dividing forms (epimastigotes) transform into metacyclic infective trypomastigotes in the insect's midgut. While biting, infected bugs deposit faeces containing metacyclic trypomastigotes on the skin (human infections occurs through the bite wound or penetration of mucous membranes of the eyes, nose, or mouth). After cell invasion, the vacuoles are disrupted and the parasite escapes into the cytoplasm of the cell, where it replicates into round-shaped amastigotes. After several binary divisions, infective trypomastigotes are released into the blood and tissue spaces. Chagas' disease affects primarily the heart and the nervous system. After a brief acute phase, patients develop a chronic infection resulting in neurological disorders, in the formation of mega-organs (megacolon or megaesophagus) and in the damage of the heart muscle. Presently available therapeutics (nifurtimox and benznidazole) show limited efficacy and severe side effects for the treatment of chronic forms of the disease. Efforts to characterize novel drug targets in *T. cruzi* identified a papain-like cysteine protease, termed cruzipain (also known as cruzain).[205−207] Cruzipain is the major proteolytic enzyme present in all stages of the life cycle of the parasite.[206,208] Biotin-labeled peptidyl diazomethane inhibitors of cruzipain revealed the highest expression levels in the epimastigote forms.[209] Cruzipain is encoded by numerous polymorphic genes organized in tandem units (up to 130 in the Tul2 strain), resulting in relative complex isoforms with a substrate specificity between those of cathepsins L and B.[210−212] Inhibition of cruzipain has been shown to impair in vitro host cell invasion and to block amastigote replication as well as trypomastigote-amastigote differentiation, thereby arresting intracellular development.[81,213−215] More recently, a novel class of irreversible cysteine protease inhibitors, vinyl sulfones, induced an accumulation of the proform of cruzipain in the Golgi apparatus resulting in the death of *T. cruzi* epimastigotes[216] (see example 39 in Table 2). In vitro studies demonstrated that cruzipain is involved in the activation of the kinin cascade, favoring parasite invasion in the host cells expressing kinin receptors.[190,217] Several crystal structures of cruzipain-inhibitor complexes have been published. They are presently the only structures solved among parasite cysteine proteases. The structure of cruzipain revealed the presence of a Glu[205] residue (papain numbering) at the bottom of the S2 pocket, the major determinant of substrate specificity.[218] Similarly to human cathepsin B which contains Glu at the same position, the cruzipain S2 specificity pocket is able to bind both P2 arginine and phenylalanine residues.[219] To facilitate the design of specific inhibitors, the substrate specificity of this protease was mapped by screening fluorogenic substrate libraries with proteogenic and nonproteogenic amino acids. Data

obtained showed that cruzipain accepts bulky hydrophobic aromatic amino acids (Phe, Tyr) as well as nonaromatic (3-cyclohexyl-alanine) residues in the P2 position. Arg or Leu are preferentially accepted in the P1 position, whereas positions P3 and P4 are less specific. Contrary to mammalian cathepsins L and B, cruzipain accommodates a proline at P2′.[220−224]

3.2.3. African Trypanosomiasis

Most of the African trypanosomes are transmitted by the tsetse fly vector (*Glossina sp*.). The species that cause human African trypanosomiasis, also known as "sleeping sickness", are represented by two main subspecies of *trypanosoma*: *T. brucei rhodesiense* and *T. brucei gambiense*. It has been estimated that at least 50 million people are at risk to be infected with trypanosomes (http://www.who.int/tdr/diseases/tryp/default.htm). African trypanosomes also infect wild and domestic animals (*T. congolense*, *T. vivax*, and *T. brucei brucei*) responsible for "nagana" in cattle which can be transmitted to humans from these animals. It has been recorded that nagana may affect more than 40 million cattle resulting in severe production losses in livestock and financial losses between $6 and 12 billion per year.[225] In humans, infections with these parasites result in severe clinical manifestations or a lethal outcome if untreated. As opposed to *T. cruzi*, the life cycles of most African trypanosomes are exclusively extracellular. The African trypanosomes multiply by binary division in the blood stream. They are able to escape from host immune response attack by altering their surface antigens (variant surface glycoprotein) and starting a new multiplication cycle if the infection is untreated. Clinical manifestations of African trypanosoma infections are intermittent fever, headaches, maniacal behavior, transient edema, and excessive sleepiness. Infections especially affect the myocardium and central nervous system generating a chronic disease with generalized lymphadenopathy and often fatal meningoencephalitis. If untreated, death usually occurs within a few years. Facing the dramatic resurgence of trypanosomiasis in Africa and the toxicity and limited efficacy of current drugs, new chemotherapies are necessary. Over the past decade, studies have demonstrated the key role of trypanosomatids thiol-proteases in rational drug or vaccine design. In African trypanosomes, three major cysteine proteases have been studied: rhodesain from *T. b. rhodesiense* and its equivalent form in cattle, congopain from *T. congolense* and brucipain, from *T. b. brucei* (also known as trypanopain-Tb). All three enzymes promote lysosomal activities and have been identified in all life-cycle stages of the protozoa, particularly during the infective stage of parasite development.[33,226,227] The molecular masses of the zymogens are between 33 and 40 kDa and they share amino acid sequences and biochemical characteristics similar to cruzipain and mammalian cathepsin L. Like cruzipain, the *T. congolense* enzymes are characterized by the presence of an unusual long glycosylated C-terminal extension, linked to the catalytic domain by a hinge region rich in proline residues in *T. congolense* (threonine-rich in cruzipain).[193,228−230] It has been suggested that the C-terminal extension contributes to the high antigenicity of these proteases.[231,232] The inhibition of the cysteine protease by small synthetic inhibitors was shown to kill *T. brucei* trypanosomes in culture and in animal models[233] corroborating the validity of trypanosoma cysteine proteases as therapeutic drug targets. As the major proteolytic enzymes in the trypanosomatid lysosome compartment, they are likely to play a role in the degradation of phagocytozed host proteins. Unlike their mammalian counterparts, cysteine proteases of American or African trypanosomes are stable at neutral and alkaline pH values suggesting an additional extracellular function.[33]

Recently, it has been suggested that congopain may play a role in anemia and immunosuppression in African cattle (see review, ref 230). Antibodies against congopain have been shown to provide immunological protection against parasite infection in cattle.[234] The substrate specificity of congopain (*T. congolense*) is more restricted than that of cruzipain due to the presence of Leu[205] in the S2 substrate binding pocket.[235] Interestingly, the congopain S2 pocket is very similar to the appropriate binding site in cathepsin K displaying the rather unusual preference for proline residues at P2.[230]

3.2.4. Leishmaniasis

Trypanosomatid flagellated protozoa of the genus *Leishmania* are the etiological agents of human leishmaniasis. *Leishmania* species are present worldwide in tropical and several temperate climate zones and infect about 12 million people annually (http://www.who.int/tdr/diseases/leish/default.htm). In the last decade, new cases were reported in southwestern Europe.[168] The life cycles of these protozoa involve a blood sucking sand fly as vector that transmits the parasite to vertebrate hosts. Within the vector, the parasite motile promastigote forms are present and reproduce asexually in the gut of the fly. The promastigotes enter cells of the vertebrate host and change into non-motile amastigote forms that live and replicate in mammalian macrophages. Infections with related species of *Leishmania* cause either visceral (*L. donovani*, *L. chagasi*), cutaneous (*L. tropica*, *L. major*, *L. mexicana*) or mucocutaneous diseases (*L. braziliensis*). Left untreated, the visceral forms result in high rates of mortality and there are no vaccines available for this disease.[236] The problem of leishmaniasis deals with the inadequacy of current chemotherapies as previously mentioned for other protozoal infections. An attractive potential target for new chemotherapies is a family of cathepsin L- and B-like proteases found in all species of *Leishmania*. They are predominantly expressed and active in the amastigote forms and to a lesser extent in promastigotes.[237,238] The genomic organization of the cysteine protease gene isolated from *L. mexicana* consists of 19 tandemly repeated genes encoding isoenzymes similar to mammalian cathepsin L.[183] Similar genomic organizations of cathepsin L-like cysteine protease genes were described in *L. donovani* and *L. chagasi*.[239] Cysteine protease inhibitors inhibit the growth and replication of *L. major*[237,240] and of *L. mexicana*[241,242] and more generally block the interac-

tion with the mammalian host.[243] For instance, cathepsin B-like cysteine protease-deficient mutants of *L. mexicana* revealed a significant reduction of the infection rate of macrophages.[244] In *Leishmania*, three types of cysteine protease have been identified. Type I (cathepsin L-like) proteases are present at the amastigote stages of *L. major* and *L. mexicana* and are encoded by a multiple gene copy with an unusual *C*-terminal extension, characteristic of trypanosomatid cysteine proteases from *T. cruzi*, *T. congolense*, and *T. brucei*.[238] Type II (cathepsin L-like) cysteine proteases, present at the amastigote and in promastigote stages, are encoded by a single copy[245,246] and have been described in *L. pifanoi*, *L. major*, and *L. mexicana* species.[247] Type III cathepsin B-like proteases have been described in *L. mexicana*[248,249] and in *L. major* with 82% amino acid sequence identities with each other.[238] However, despite the sequence and structural similarities (45% sequence identity) with human cathepsin B, the cathepsin B-like protease in *L. major* is not able to cleave synthetic substrates with an arginine residue in position P2. Instead of the cathepsin B glutamic acid residue at position 205, the *L. major* protease has a glycine residue.[250] Replacing the glycine by a glutamic acid residue by site-directed mutagenesis restored cathepsin B-like substrate specificity, confirming that the structurally cathepsin B-like protease of *L. major* has the P2 substrate specificity of cathepsin L-like enzymes.[251] A significant reduction of macrophage infectivity was observed with mutants lacking either type I or II or both genes indicating the importance of these enzymes as virulence factors in the physiopathology and development of leishmaniasis.[243,252,253] Surprisingly, besides its homology to cruzipain, the type II protease from *L. mexicana* has no kininogenase activity.[222] Substrate specificity studies revealed a preference for phenylalanine and leucine in P2, arginine and lysine in P3, P1′, and P3′ while the other position appeared to be nonspecific.[254,255] Subsites S2, S1 of the type II cysteine protease were explored in more detail using fluorescent substrates containing non-proteogenic basic amino-acid branched with an aromatic or hydrophobic group.[256,257] Only two substrates containing residues 4-aminomethyl-cyclohexyl-alanine (Ama) or 4-aminocyclohexyl-alanine at the P1 position were resistant to proteolysis and inhibited the enzyme with K_i values < 30 nM. As determined for cruzipain, aromaticity in P2 position is not necessary for S2/P2 interaction since Ama was well accepted in the S2 pocket of the recombinant cysteine protease from *L. mexicana*. On the basis of these data, a new combinatorial library of peptide ketone inhibitors was screened against the protease.[258]

3.2.5. Giardiasis

The flagellated protozoan *Giardia lamblia* is the most common intestinal human parasite found throughout the world, especially in Asia, Africa, and Latin America. It is the etiological agent of gastrointestinal giardiasis responsible for chronic diarrhea and malabsorption. About 200 million people have symptomatic giardiasis. In the U.S., 2.5 million new infections are reported annually (http://www.tulane.edu/~wiser/protozoology/notes/intes.html#giardia; http://www.cdc.gov/epo/mmwr/preview/mmwrhtml/ss4907a1.htm). Most *G. lamblia* infections result from ingestion of contaminated water or food, containing a resistant cyst form of trophozoites. The cystic form allows the parasite to survive outside the body environment for long periods of time. Infection is initiated by the release of the motile trophozoites from the cyst in the host and this excystation is mediated by cathepsin B-like cysteine proteases.[259] It has been shown that cysteine protease inhibitors can prevent excystation.[26] However, the proteases do not seem to be involved in the motility or the replication of the trophozoites.[259] Three cathepsin B-like proteases (GlCP1, GlCP2, and GlCP3) have been described in the cytoplasmic vacuoles of *G. lamblia*. Interestingly, these proteases do not possess the occluding loop characteristic of mammalian cathepsin B. In contrast, other cathepsin B-like proteases from protozoa and helminth parasites possess comparable occluding loops although they reveal various deletions or insertions of amino acids. To date, the effect of these loop modifications has not been studied in detail with respect to the endo/exopeptidase activity of the parasitic proteases.

3.2.6. Trichomoniasis

Trichomonas vaginalis is a flagellated protozoan which infects the urogenital system, responsible for vaginitis and urethral discharge (trichomoniasis). Trichomoniasis is the most prevalent sexually transmitted disease, affecting several million people every year, particularly in Africa and North America.[260] *T. vaginalis* infection may also increase the rate of HIV transmission by degrading the secretory leukocyte protease inhibitor.[261] Contrary to giardiasis, there is no cystic form in the life cycle, so transmission is via the trophozoite stage. Cervicovaginal epithelium colonization through the mucus layer requires the adherence of the parasite and extracellular matrix degradation implicating the role of proteolytic activities.[262] 2-D gel electrophoresis analysis revealed 23 different cysteine proteases with high molecular masses up to 96 kDa in *T. vaginalis*.[263] Some of the proteases participate in cytoadherence,[262,264] hemolysis,[265] cytotoxicity,[266] and immunoevasion.[267] They are able to degrade C3 complement and some extracellular matrix proteins, i.e., collagen IV and fibronectin. Another immunogenic cysteine protease has been described (CP30) which is secreted into the vagina during infection and is involved in cytoadherence.[268] Cell culture experiments using peptidyl acyloxymethyl ketone inhibitors indicated that cysteine protease inhibitors can block *T. vaginalis* growth.[269]

A cathepsin B-like protease with a type I collagenolytic activity has been isolated from *T. tenax*, responsible for pulmonary trichomoniasis.[270,271]

3.2.7. Amoebiasis

Entamoeba histolytica, an invasive endoparasite, and *E. dispar*, a noninvasive ectoparasite infect more than 50 million people worldwide causing amoebic dysentery (see review, ref 189). *E. histolytica* penetrates the host's intestinal walls and also affects the

liver where it can destroy liver parenchyma cells and produce large abscesses. If left untreated, amoebiasis can be fatal and causing mortality in over 70,000 people annually. Amoebiasis constitutes the third most frequent cause of death by parasitic infections after malaria and schistosomiasis. The life cycle of *E. histolytica* involves motile trophozoites (the feeding stage of the parasite) and infections occur ingesting the amoebic cyst in contaminated food or water. Excystation and invasion of the parasite into the host tissue require the proteolytic activity of cysteine proteases. The proteases are involved in the degradation of the intestinal mucoproteins and extracellular matrix proteins.[272] In addition, cysteine proteases from *E. histolytica* have been shown to be virulence factors[273] and are highly immunogenic proteins in the serum of infected patients.[189] A purified cysteine protease activity from *E. histolytica* was able to cleave human IgA, IgG and specifically the complement components C3 and C5 leading to a circumvention of host immunity.[274] Two enzymes named amoebapain or EhCP1[275] and histolysin[272] have been characterized. Despite their high amino acid sequence identity (87%), they display distinct activities. To date, three other cysteine protease genes have been identified in *E. histolytica*. All gene products shares amino acid sequences identities between 36 and 46% with human cathepsin L.

One major 30 kDa cathepsin L-like cysteine protease was identified from a related amoeba, *Naegleria fowleri* which is responsible for amoebic meningoencephalitis in humans and may cause in rare cases death.[276] This amoeba infects the central nervous system via the nasal mucosa and is responsible for an acute hemorrhage in the brain. In vitro, irreversible thiol-protease inhibitors abolished the degradation of extracellular matrix proteins by *Naegleria* trophozoites thus potentially acting as anti-invasive agents.[276]

3.2.8. Schistosomiasis

Chronic human schistosomiasis or often known as Bilharzia is by far the most important trematode (blood fluke) infection. There are a number of species of schistosomes that can infect humans, but most human infections are caused by *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. These parasites are distributed in tropical regions, afflicting about 200−500 million people and resulting in 1 million deaths each year, the second most deadly parasite disease after malaria (http://www.who.int/tdr/diseases/schisto/default.htm). Infection is initiated by contact with water containing the larva stages of the parasites (cercariae). The complex life cycles of human schistosomes are similar to each other. It includes snails as intermediate hosts and a vertebrate as a definitive host. After the cercariae penetrate the skin, the immature worms (schistosomula) enter the circulatory system and migrate into the lung and liver. After schistosomes penetrate the walls of the veins and the small intestine (*S. mansoni* and *S. japonicum*) or urinary bladder (*S. haematobium*), mature forms of schistosomes cause a significant amount of damage to the tissues of the host. They utilize hemoglobin as a major source for their growth, development and reproduction using several proteases, like cathepsins B, L, C, D, and legumain-like proteases.[191] Schistosomes also require proteolytic activities for the penetration of human skin and the evasion of immunological responses.[184] Interestingly, treatment of mice infected with schistosomes with broad spectrum cysteine protease inhibitors showed a significant reduction in worm burden, hepatomegaly, and the number of eggs produced per female worm.[277] Cathepsin L-like and cathepsin B-like activities have been described at different stages of the trematode development cycles (see review, ref 191). Two isoforms of cathepsin L (SmCL1, SmCL2) have been isolated from adult *S. mansoni*.[278,279] Apparent homologues papain-like cysteine proteases (SjCL1, SjCL2) have been described in *S. japonicum*.[280] A cathepsin B-like protease known as Sm31 (*S. mansoni*) is the major proteolytic activity secreted by adult worms. The protease has a high hemoglobinase activity and represents a highly immunogenic antigen in infected mammals.[281] Sequences of cathepsin C-like proteases have been reported in *S. mansoni* and *S. japonicum*.[282]

3.2.9. Fascioliasis

Fascioliasis or liver fluke disease is caused by the trematode, *Fasciola hepatica*, found in animals (sheep and cattle) as well as in humans. It has been estimated that more than 2 million people are infected worldwide.[283] This disease is a major health problem and also results in significant economic losses (estimated at >$200 billion per annum) in cattle and sheep livestock in Australia, Europe, China, and South America.[284] *F. gigantica* significantly contributes to the losses of domestic ruminants in Africa and the Middle East. Animals infect themselves by eating vegetation or drinking water contaminated with cercariae. The flukes (worms) migrate into the intrahepatic bile ducts where they mature and cause biliary tract obstruction and liver damage. Increasing resistance to the present chemotherapy against fasciolosis in animals asks for novel therapeutic approaches. To confer protection against liver fluke infection, cathepsin L-like proteases are good candidates for rational design of new antiparasite drugs or potential vaccines.[284] Two isoforms of cathepsin L-like proteases (FheCL1 and FheCL2) have been isolated from the excretion/secretion products of liver flukes.[285,286] They have been localized to the reproductive organs of *F. hepatica* suggesting that they may play a role in the reproduction of the parasites.[287] It has been demonstrated that they are immunodominant antigens in human fascioliasis.[288] Antibodies against cysteine proteases have shown promising results in terms of protection against parasite infection.[289] Studies showed that cysteine proteases facilitate the migration of the parasite in various host tissues by degrading tissue and matrix proteins, the utilization of host tissue as source of nutrients (e.g., hemoglobin degradation) as well as the modulation of the host immune response.[284] For instance, they are able to release kinins from human high molecular weight kininogens[290] helping the evasion of the parasite. Besides their ability to cleave

host collagen and other extracellular matrix components such as laminin, *fasciola* cysteine proteases are also capable to degrade all human IgG subclasses in the hinge region and prevent attack by host immune cells.[291−293] This function was also described for a *F. hepatica* cathepsin B-like protease.[294]

3.2.10. Paragonimiasis

Infection with adult forms of the trematode, *Paragonimus westermani*, may cause chronic inflammatory lung disease mimicking tuberculosis in humans. The most important endemic areas are in Asia, West Africa, South and Central America. About 20 million people became infected by eating crabs or crayfish infected by metacercariae (http://www.who.int/ctd/intpara/burdens.htm). The metacercariae migrate in the digestive tract, and finally enter lung tissue and develop into adult worms. Worms may also develop in the brain, causing paraplegia or seizures. While invading host intestine, several different secreted cysteine proteases have been identified (≥ 6) in different maturation stages of the trematode. They participate in a broad range of biological processes such as tissue migration and immune evasion.[295] Similar to *G. lamblia*, excystment of the lung fluke is modulated by cysteine protease inhibitors. One cathepsin L-like cysteine protease (PWCP1) has been localized mainly in the vitelline gland of the worms and may therefore participate in the biosynthesis of *P. westermani* eggs.[296] Its amino acid sequence is highly homologous to human cathepsin F (49% identity).[297] In addition, PCWP1 possesses a long proregion of 210 amino acids which contains the motif DRFNMQ (instead of ERFNAQ in cathepsin F) and also a cystatin-like domain. These data suggest that PCWP1 belongs to the cathepsin F subfamily of papain-like thiol proteases.

3.2.11. Intestinal Nematodes (Roundworms) Infections

Several species of nematodes are responsible for intestinal roundworms infections. Among them *Ascaris lumbricoides* is one of the largest and most common parasitic nematode (ascarids) found in human intestines. It has been estimated that up to 25% of the world's population is infected with this nematode and that approximately 200 million of the cases are symptomatic (http://www.biosci.ohio-state.edu/~parasite/ascaris.html). The parasite is responsible for pneumonia caused by the worm's migration through the lungs. It also affects the gastrointestinal track or the bile or pancreatic ducts by blocking them. The life cycle of *A. lumbricoides* is similar to *Ascaris suum* (pig) and consists of the ingestion of contaminated food or water or through contact with infected soil. Nematodes grow and develop through four molts, the last two in the final host. In the small intestine, the eggs transform into juvenile forms and penetrate the small intestine before entering the circulatory and respiratory system. Serious damages occur during the migration of the worms causing hemorrhages in the lung. The juvenile worm then migrates up into the pharynx to be re-swallowed again, and returning to the small intestine where the adult worms mature. Numerous cathepsin B-like protein sequences have been published or reported in ascarids[298,299] and other parasitic nematodes such as the hookworms *Ancylostoma duodenale*, *A. caninum*,[300] *Necator americanus*, the whipworm *Trichuris trichiura* and *T. suis*,[301] the intestinal threadworm *Strongyloides stercoralis*, *S. ratti*,[302] *Ostertagia ostertagi*,[303] *Onchocerca volvulus*,[304] and *Nippostrongylus brasiliensis*.[305]

Metallo- and serine proteases are also expressed by these parasitic nematodes and are thought to play an essential role in the molting of the third (L3) stage larvae, digestion, and facilitating tissue migration.[306] Cysteine proteases are involved in larval invasion at different stages of these parasites.[26] In vitro, thiol-protease class specific inhibitors blocked the development of L3 stage larvae of *A. suum*, affecting molting.[299] Experiments also showed that cysteine proteases (similar to cathepsin C or cathepsin L) of the filarial nematode *Onchocerca volvulus* are located in the cuticula surrounding the molting L3 larvae stage.[304]

Ancylostoma caninum, which induces human eosinophilic enteritis, contains two cysteine proteases (AcCP1 and AcCP2) belonging to the cathepsin B-like subfamily. They share a 61% amino acid sequence identity with *Haemonchus contortus* and about 50% with human cathepsin B. The proteases are expressed in the esophageal, amphidial and excretory glands in adult hookworms[300] and like *N. americanus*[307] are responsible for necatoriasis (see for review, ref 308). Cysteine protease activities were detected in excretory/secretory products of the live third-stage larvae of *N. americanus*[309] and were able to cleave immunoglobulins. Hemoglobulin-degrading activity in the pH range 5 to 7 and fibrinogenolytic activity at pH 3.5 was detected.[310] A hemoglobinolytic activity was also reported in adult *Ascaris suum*.[298] *T. suis* adult worms expressed a cathepsin B-like activity in the gut which was also found as a secreted form suggesting a role in feeding and digestion.[301]

While cathepsin B-like cysteine proteases are prominent among nematodes, cathepsin L-like enzymes are less common. However, genes encoding cathepsin L- and S-like proteases have been described in a cDNA library of the plant parasitic nematode *Heterodera glycines*.[311]

Toxocara canis, the etiological agent of toxocariasis, expresses a cathepsin L-like activity (TcCP-1) in its excretory/secretory products which shares a 69% amino acid sequence identity with the cathepsin L found in the filarial parasite *Brugia pahangi*.[312] It is an immunogenic antigen which could serve as a diagnostic marker for human toxocariasis. Like cruzipain, TcCP1 shares a substrate specificity with cathepsin B due to a Glu^{205} residue at the bottom of the S2 substrate binding pocket. Interestingly, AcCP1 protease from *A. caninum*, a cathepsin B-like enzyme at the sequence level, exhibits a cathepsin L-like specificity with a preference for aromatic residues in the P2 position. A glutamine residue is found a position 205 in its S2 pocket.[313] Beside the nematode *Caenorhabditis elegans*, one of the best characterized worms is *Haemonchus contortus*.[314−316] Four genes encoding cathepsin B-like proteases in *H. contortus*

exhibit cathepsin L-like substrate specificities by lacking the residue Glu[205] in the S2 subsite pocket.[317,318] Immunocytochemical studies localized these proteases to the microvillar intestinal tissue of the parasite's gut and during the blood-feeding parasitic stages exclusively in its excretory/secretory products.[319] In vitro experiments showed the uptake and digestion of hemoglobin[320] and the degradation of extracellular matrix proteins such as collagen and elastin.[321] Several other uncharacterized genes (at least 11 more genes) encoding cathepsin B-like proteases have been identified in *H. contortus*.[322]

Recently a cathepsin X-like cysteine protease gene was found in adult and infective stages of larvae *T. canis* with 59% protein sequence identity with human cathepsin X.[323] Human infections by the cestode *Spirometra* leading to sparganosis involved a cathepsin L-like protease which may be involved in the pathogenesis of this tapeworm, including tissue penetration, nutrition, and immunoevasion in vitro (see for review, ref 184).

4. Recent Developments in Inhibitors Design for Cysteine Proteases

The activity of papain-like cysteine proteases is regulated by a large number of endogenous protein-based inhibitors. Two major inhibitor classes are known: (i) cystatins and (ii) serpins which are active toward cysteine proteases. Among the cystatins, intracellularly, extracellular-transcellularly and intravascularly expressed inhibitors can be distinguished. Cystatins have been implicated in a wide range of regulatory and disease-related processes such as in immune responses,[324] cancer,[141,325] and immune evasion by parasites.[326] More recently, members of the serpin inhibitor family (serpin: *ser*ine *p*rotease *in*hibitors) have been demonstrated to show cross-reactivity toward cathepsins (see for review, ref 327). It is, however, not in the objective of this review to discuss the natural cysteine protease inhibitors in detail. The interested reader is referred to various reviews discussing the roles of endogenous cysteine protease inhibitors in health and disease as well as structure−function issues.[328−331]

The design and synthesis of cysteine protease inhibitors has a long history and has been extensively reviewed in recent years.[80,329,332−336] Compounds synthesized included a wide range of peptide aldehydes, methyl ketones, nitriles as reversibly acting inhibitors and diazomethanes, halomethyl ketones, acyloxymethyl ketones, *O*-acylhydroxamates, and epoxysuccinyl derivatives as irreversible inhibitors. Whereas early developments of cysteine protease inhibitors provided useful tools to study the protease activity, only recently significant progress has been accomplished to develop cysteine protease inhibitors into drugs. This development was mostly driven by the dramatic increase in our understanding of papain-like cysteine proteases as pharmaceutically valid targets as outlined above. This section will concentrate on recent developments in cysteine protease inhibitor drug design and examples for each inhibitor class are compiled in Table 2.

4.1. Diacyl Bis Hydrazides

Diacyl bis hydrazides evolved from previously reported diaminopropanones which have been developed as potent and selective cathepsin K inhibitors spanning both S and S′ subsites of the substrate binding cleft.[99] The incorporation of bis aza analogues increased the potency while maintaining the selective profile for cathepsin K. The most potent compounds possess a leucine residue either in the P2 or P2′ position or in both (see Table 2; examples 1, 2, and 4). Example 4 is a reversible cysteine protease inhibitor which contains a peptidomimetic thiazole ring in place of an amide bond and may form an acyl adduct with the enzyme. In contrast, the acyclic examples 1, 2, and 3 are irreversible inhibitors of cathepsin K. The importance of a proper S2P2 and S2′P2′ binding interaction was demonstrated by the replacement of the leucine residues by alanine residues (example 3). The second-order rate constant of the alanine-containing derivative dropped by more than 2-orders of magnitude with most of the loss due to a dramatically reduced affinity of the compound.[97] In contrast, replacements of the P2′ leucine residue with other hydrophobic residues such as norvaline (Table 2; example 5) or with a non-amino acid aromatic moiety (Table 2; example 6) retained the potency of the compounds.[337] Example 1 was evaluated in a human osteoclast resorption assay and showed efficacy with an IC50 value 0.34 μM. A slightly modified version of example 1 was tested in an acute model of bone resorption (TPTX rat model) in vivo and caused a highly significant inhibition of the osteoclast-mediated calcemic response to hPTH.[97]

4.2. Diamino Pyrrolidinone

Above described diacyl hydrazides and cyclic thiazole ring analogues were susceptible to acylation which may lead to the proteolytic cleavage of the inhibitors. Therefore, the bis hydrazide moiety was modified by the incorporation of five-membered rings under maintaining the P2 and P2′ isobutyl structures (Table 2; example 7). This compound retained the nanomolar affinity toward cathepsin K. However, the removal of the P2 isobutyl chain increased the K_i value 10-fold iterating the importance of P2S2 hydrophobic interactions (Table 2; example 8).[338]

4.3. Aldehydes

Aldehydes and its analogues continue to be attractive moieties despite their well-established chemical reactivity. Recent efforts in designing aldehyde-based inhibitors are focused on achieving high selectivity toward cathepsins. For example, potent tripeptidyl leucine analogues (Table 2; example 9) targeting cathepsin K have been evaluated in rat adjuvant arthritis and PTH induced osteoporosis models.[339]

A potent and selective cathepsin S inhibitor was prepared by incorporating an α-keto moiety with a nontypical P2 requirement for cathepsin S, phenylalanine (Table 2; example 10). Against expectation, this compound proved to be very potent and selective for cathepsin S when compared with cathepsin B (400-fold potency difference). The kinetic charac-

terization suggested a reversible, slow binding mode of inhibition.[340] Along these lines, the introduction of an acetylated lysine residue in P2 (Table 2; example 11) resulted in an additional potent and selective cathepsin S inhibitor which was active in the nanomolar concentration whereas the inhibition of cathepsin B and L required micromolar inhibitor concentrations.[341] This compound has been recently tested in a murine model of Sjögren syndrome and proved efficacious.[156]

Peptidomimetic derivatives of monopeptidyl aldehydes were potent and selective cathepsin L inhibitors and showed in vivo efficacy in a mice model of bone resorption. Examples with isoleucine, leucine, and alanine were similarly potent (Table 2; examples 12, 13, and 14). A significant loss of activity was observed with a glycine-substituted derivative.[342]

Katunuma et al.[343] introduced synthetic aldehyde analogues of vitamin B6 as cathepsin inhibitors. Surprisingly, the replacement of the phosphate ester in vitamin B6 by propionate resulted in a modestly potent cathepsin B inhibitor (Table 2; example 15). The extension of the example 15 with a isoleucine moiety (Table 2; example 16) improved significantly the potency and provided a cathepsin K selective inhibitor. The compound also demonstrated efficacy in an in vitro bone resorption assay.

Since cysteine and metalloproteinases have been implicated in tumor invasion and metastasis, double-headed inhibitors would be desirable. Yamamoto et al.[344] synthesized inhibitors which have an aldehyde moiety for cathepsin inhibition and a chelating hydroxamic acid portion for the inhibition of matrix metalloproteinases (MMP). These dual inhibitors displayed remarkable activities toward both protease classes in the nanomolar concentration range. Depending on the amino acid substitutions used, the selectivity of the compounds could be either tilted toward cathepsin or MMP activity. An alkyl group at the α-carbon of the aldehyde moiety was essential for maintaining cathepsin L potency (Table 2; examples 17−20).

4.4. Acyclic and Cyclic Ketones

Attempts to reduce the high reactivity of the aldehyde group led to the development of acyclic and cyclic ketone derivatives. α-Substituted ketone dipeptides revealed potency toward cathepsin K which could be modulated by modifications of the ketone moiety. K_i values in the low nanomolar range could be achieved with a dipeptidyl phenyloxymethyl ketone (Table 2; example 21) whereas an appropriate methoxymethyl ketone (Table 2; example 22) resulted in a 20-fold decrease in affinity.[345]

The design of mercaptomethyl ketones as cruzipain inhibitors (Table 2; example 23) was based on specificity information from diazomethyl ketones and vinyl sulfones (see section 4.8.) inhibitors. Several modifications of the structures such as the incorporation of pyridine, homophenylalanine, and leucine to improve solubility and bioavailability while maintaining selectivity and potency, proved to be successful. Example 23 displayed a greater than 100- or 1000-fold selectivity for cruzipain when compared with cathepsins B or L, respectively.[346]

Conformationally constrained cyclic ketones were aimed to lock the bioactive conformation of the compounds and to improve bioavailability. The cyclic conformers did not lose potency toward cathepsin K (Table 2; example 24) and thus provided a useful scaffold for further modifications. The inhibitors covering extended binding sites of cathepsin K were significantly more potent than the single site analogue (compare examples 24 and 25). The introduction of a sulfonamide moiety in combination with a six membered ring oxy ketone resulted in a very potent and selective cathepsin K inhibitor (Table 2; example 26).[347,348]

The introduction of an azepanone moiety to prevent epimerization of the chiral center in the cyclic ketone analogues resulted in a further improvement of the potency into the subnanomolar range for human cathepsin K and in increased bioavailabilities of the compounds (Table 2; example 27).[349] Example 27 was tested in a nonhuman primate model of osteoporosis and resulted in a significant reduction in serum markers of bone resorption relative to untreated controls. After 5 days of dosing, the reductions in N-terminal telopeptides (NTx) and C-terminal telopeptides (CTx) of type I collagen were 61 and 67%, respectively. This is the first example using nonhuman primates to evidence the viability of cathepsin K inhibitors as alternative treatment of postmenopausal osteoporosis.[96]

4.5. Nitriles

Recently, nonpeptidyl derivatives of nitriles employing pyrrolidine or azetidine rings have been demonstrated to be potent cysteine protease inhibitors. Interestingly, a four-membered ring derivative (Table 2; example 29) was approximately 10-fold more potent than the five-membered ring analogue (Table 2; example 28) which was possibly due to the increased chemical reactivity of the azetidine ring. Nonpeptidyl nitriles act as reversible, but time-dependent inhibitors by forming a cleavable isothiourea ester link with the enzyme.[350]

New dipeptidyl cyanamides targeting cathepsin B revealed IC50 values in the low nanomolar range whereas cathepsins L and S required approximately 100-fold higher inhibitor concentrations for a comparable inhibition. These compounds inhibited cathepsin B with a reversible mode of inhibition by forming an unstable thioimidazole ester linkage with the active site cysteine residue of the protease (Table 2; example 30).[351]

4.6. Epoxysuccinyl Analogues

Structural information about the reverse mode of binding of the propeptide to active cathepsin B was utilized in designing novel, extended active site spanning E-64 peptidyl analogues. Selective cathepsin B or cathepsin L inhibitors were synthesized with the most active compounds spanning over six subsites (P4 to P2′ residues). The most potent

Table 2. Novel Inhibitors of Papain-Like Cysteine Proteases

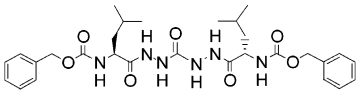
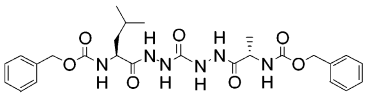
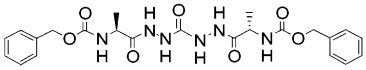
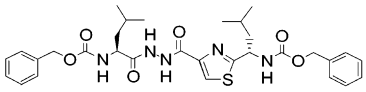
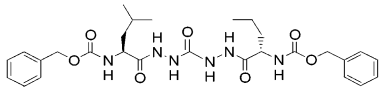
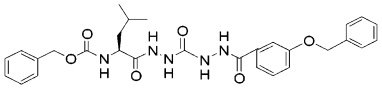
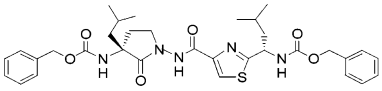
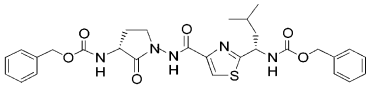
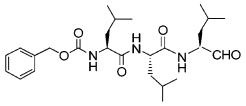
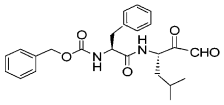
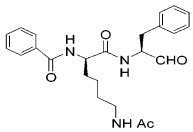
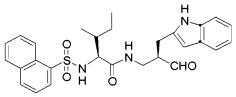
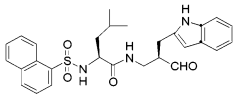
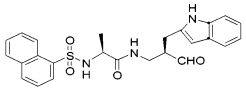
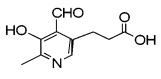
	Chemical Class	Structure	Activity	Mode of inhibition	Ref.
1	diacyl - bis hydrazide		cat K k_{obs} /[I]=3.1x10^{6} M^{-1} s^{-1} Ki_{app}=0.7 nM cat S Ki = 11 nM potent cat K and S inhibitor	irreversible	97
2	diacyl - bis hydrazide		cat K k_{obs} /[I] =6.9x10^{5} M^{-1} s^{-1} Ki_{app} = 1.0 nM cat S Ki = 44 nM potent cat K inhibitor	irreversible	97
3	diacyl - bis hydrazide		cat K k_{obs} /[I] =2.2x10^{3} M^{-1} s^{-1} Ki $_{app}$> 1000 nM loss of potency	irreversible	97
4	bis hydrazide - cyclic analog		cat K Ki_{app}= 10 nM cat B Ki_{app}= 5200 nM cat L Ki_{app} =700 nM cat S Ki_{app}>1000 nM selective cat K inhibitor	reversible	97
5	diacyl- bis hydrazide		cat K K_{obs}/[I] = 4.0x10^{6} M $^{-1}$s^{-1} Ki_{app} =0.8 nM potent cat K inhibitor	irreversible	337
6	diacyl - bis hydrazide peptidomimetic		cat K K_{obs}/[I]= 3.2x10^{5} M^{-1}s^{-1} Ki_{app} = 12 nM potent cat K inhibitor	irreversible	337
7	diamino pyrrolidinone		cat K Ki = 33 nM potent cat K inhibitor	unknown	338
8	diamino pyrrolidinone		cat K Ki = 330 nM	unknown	338
9	tripeptidyl aldehyde		cat K Ki_{app} = 1.4 nM potent cat K inhibitor	reversible	339
10	α keto aldehyde		cat S Ki =0.185 nM cat B Ki = 76 nM selective cat S inhibitor	reversible slow binding	340
11	aldehyde		cat S 40% inh.at 10 $^{-8}$ M 86% inh. at 10 $^{-7}$M selective cat S inhibitor	possibly reversible	156, 341
12	aldehyde		cat L IC_{50}= 1.9 nM cat B IC_{50} = 1500 nM selective cat L inhibitor	reversible	342
13	aldehyde		cat L IC $_{50}$ = 2.2 nM cat B IC $_{50}$ >1000 nM selective cat L inhibitor	reversible	342
14	aldehyde		cat L IC $_{50}$ = 5.3 nM cat B IC $_{50}$ = 530 nM selective cat L inhibitor	reversible	342
15	pyridoxal propionate aldehyde		cat B 70% inh. at 10 $^{-5}$ M	possibly reversible	343

Table 2 (Continued)

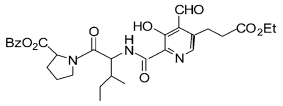
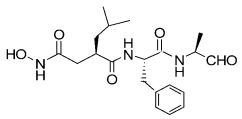
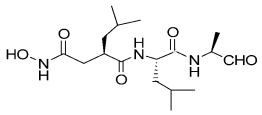
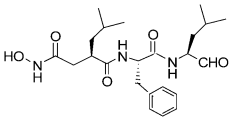
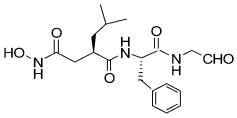
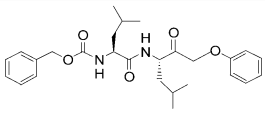
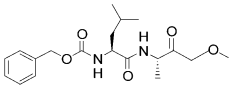
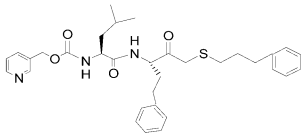
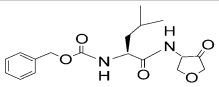
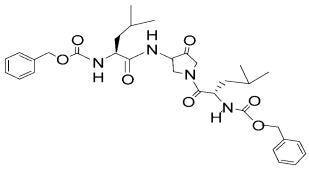
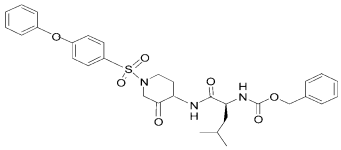
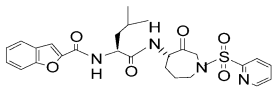
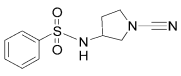
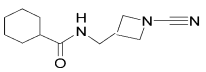
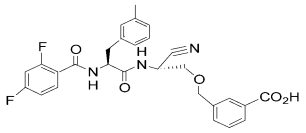
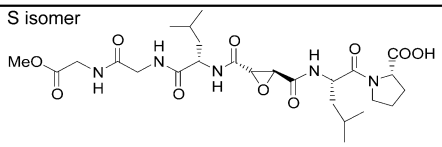
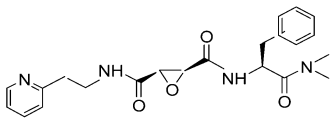
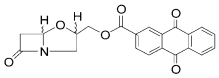
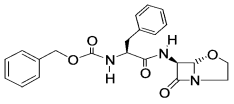
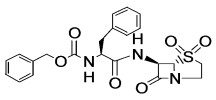
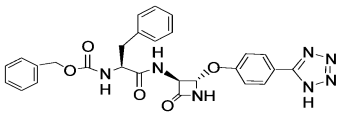
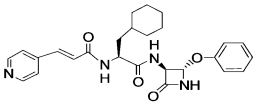
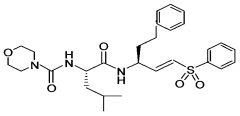
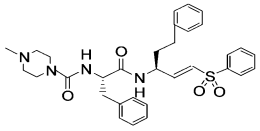
	Chemical Class	Structure	Activity	Mode of inhibition	Ref.
16	pyridoxal propionate aldehyde		cat K 60% inh.at 10^{-6} M 40% inh at 10^{-7} M selective cat K inhibitor	possibly reversible	343
17	aldehyde - hydroxamic acid combination		cat L IC_{50} = 15 nM MMPI IC_{50} = 25 nM dual MMP and cat L inhibitor	possibly reversible	344
18	aldehyde - hydroxamic acid combination		cat L IC_{50} = 23 nM MMPI IC_{50} = 47 nM dual MMP and cat L inhibitor	possibly reversible	344
19	aldehyde - hydroxamic acid combination		cat L IC_{50} = 7 nM MMPI IC_{50} = 1400 nM dual MMP and cat L inhibitor	possibly reversible	344
20	aldehyde - hydroxamic acid combination		cat L IC_{50} = 4300 nM MMPI IC_{50} = 12 nM loss of cat L potency	possibly reversible	344
21	dipeptidyl ketone		cat K Ki_{app} = 8.0 nM potent cat K inhibitor	irreversible	345
22	dipeptidyl ketone		cat K Ki_{app} = 150 nM	not known	345
23	mercapto methyl ketone		cruzipain Ki = 1.1 nM cat B Ki = 1700 nM cat L Ki = 144 nM selective cruzipain inhibitor	slow binding moderately time dependent	346
24	cyclic ketone		cat K Ki app = 140 nM	reversible competitive	347
25	cyclic ketone		cat K Ki app = 2.3 nM cat L Ki app = 39 nM cat S Ki app = 90 nM selective cat K inhibitor	reversible competitive	347
26	cyclic ketone peptidomimetic		cat K Ki_{app} = 0.5 nM cat B Ki_{app} > 1000 nM cat L Ki_{app} > 1000 nM selective cat K inhibitor	transition state analog	348
27	azepanone analogs		cat K Ki = 0.16 nM cat L Ki = 2.2 nM cat S Ki = 4.0 nM selective cat K inhibitor	reversible	96, 349
28	non peptidyl nitrile		cat K and L IC_{50} = 50 and 80 nM respectively	reversible time dependent	350

Table 2 (Continued)

	Chemical Class	Structure	Activity	Mode of inhibition	Ref.
29	non peptidyl nitrile		cat K and L IC_{50} = 5 and 6 nM respectively	reversible time dependent	350
30	dipeptidyl nitrile		cat B IC 50 = 6.8 nM cat L IC 50 = 554 nM cat S IC 50 = 937 nM selective cat B inhibitor	reversible	351
31	E 64 analog	S isomer 	cat B k_2/Ki =1.5x10^6 M^{-1} s^{-1} cat L k_2/Ki =1.2x10^3 $M^{-1}s^{-1}$ selective cat B inhibitor	irreversible	352
32	E 64 analog		cat L 63 % inh.at 100 nM cat K and S active at 10 $^{-5}$ M selective cat L inhibitor	irreversible	341, 353
33	β lactams oxapenam		cat K IC 50 = 60 nM cat S IC 50 = 79 nM cat L IC 50 = 79 nM cat B IC 50 = 693 nM non selective	irreversible	358
34	β lactams oxapenam		cat K IC 50 = 5 nM cat S IC 50 = 77 nM cat L IC 50 = 4 nM cat B IC 50 = 12200 nM cat K and L selective	reversible	357
35	β lactams penam		cat K IC 50 = 0.5 nM cat S IC 50 = 10 nM cat L IC 50 = 1.4 nM cat B IC 50 = 350 nM cat K and L selective	slowly reversible	359
36	β lactams monobactam		cat K IC 50 = 14 nM cat L IC 50 = 110 nM cat S IC 50 =360 nM selective cat K inhibitor	reversible	360,361
37	β lactams monobactam		cat S Ki = 1 nM cat K Ki = 700 nM cat L Ki = 195 nM cat B Ki = 820 nM selective cat S inhibitor	reversible	360,361
38	vinyl sulfones		cat S k_2/Ki =2.6x10^7M^{-1} s^{-1} cat B k_2/Ki = 4.3x10^6$M^{-1}s^{-1}$ cat K k_2/Ki =7.7x10^5 $M^{-1}s^{-1}$ cat L k_2/Ki = 3.9 x10^5$M^{-1}s^{-1}$ potent, non-selective inhibitor	irreversible	362, 363
39	vinyl sulfones		cruzipain k_2/Ki = 6.5 x10^6 $M^{-1}s^{-1}$ P.falciparum IC_{50} = 5 nM potent cruzipain inhibitor	irreversible	362, 363

cathepsin B inhibitor derivative contained a Gly-Gly-Leu peptide in the P4−P2 position and a Leu-Pro-OH in the P1′−P2′ position (Table 2; example 31).[352] A similar selective and potent cathepsin L inhibitor was obtained by introducing a Phe-dimethylamide moiety into the P2 residue (Table 2; example 32). This compound was effective in reducing tumor-induced hypercalcemia in mice.[341,353]

Peptidyl epoxides with a tyrosine and biotin moieties allowing iodination and streptavidin-based de-

tection proved very valuable as functional proteomics tools.[354,355]

4.7. β-Lactams

β-Lactams are well-known as antibiotics with penicillin as its first and most known example.[356] Their common feature is a core β-lactam ring. Because resistance to β-lactam antibiotics among target pathogens developed early in the history, large numbers of β-lactam derivatives have been synthesized. The development of β-lactams as cysteine protease inhibitors is, however, very recent. Single ring as well as a bicyclic ring β-lactam moieties have been evaluated as inhibitors of cysteine proteases. The β-lactam moiety serves as thiol reactive species and is linked with nonpeptidyl or amino acid or peptide portions targeting binding site pockets of relevant cathepsins. 2-Substituted oxapenams employing a nonpeptidyl aromatic or alkyl moiety as subsite motif displayed surprisingly potent inhibitory efficacy in the mid-nanomolar range (Table 2; example 33). As expected, lacking a specific targeting moiety, these compounds were not selective. The mode of inhibition was time dependent and no recovery of enzymatic activity was observed. On the other hand, the incorporation of amino acid or peptidyl moieties as targeting sequence for individual proteases at the other side of the ring resulted in a significant enhancement of the potency and specificity of the compounds. IC50 values in the nanomolar range were observed and the selectivity of the compounds was improved (Table 2; example 34). The replacement of the oxygen in the oxapenam ring by sulfur further improved the potency (Table 2; example 35). Examples 34 and 35 show clear selectivity for cathepsins K and L over cathepsins B and S.[357−359]

Monobactams revealed a reversible mode of inhibition. Specificity knowledge based modifications of P and P′ positions in peptidyl monobactams led to the development of more selective cathepsin inhibitors. Selective cathepsin K inhibitors contained heterocyclic and aromatic rings targeting the S′ binding sites (Table 2; example 36), while a hydrophobic cyclohexane moiety targeting the S2 pocket produced cathepsin S specific inhibitors (Table 2; example 37).[360,361]

4.8. Vinyl Sulfones

Peptidyl vinyl sulfone inhibitors are remarkably potent irreversible inhibitors of cathepsins. A second-order rate constant up to 26×10^6 M^{-1} s^{-1} for cathepsin S has been described.[111,362] They have been shown to be effective in mice arthritis models by significantly reducing inflammation as well as bone and cartilage erosion (Table 2; example 38).[110] However, due to the irreversible mode of action, vinyl sulfone inhibitors are unlikely to be developed as therapeutic drugs for chronic diseases such as osteoporosis. On the other hand, their high potency against various parasitic cysteine proteases makes them ideal drug candidates for the treatment of acute and chronic infections. Of particular value was the observation that selected vinyl sulfones were completely tolerated in mice during a 45-day course of therapeutic regimen of 100 mg/kg/day.[363] Example 39 (Table 2) was demonstrated to cure *T. cruzi* infections in a mouse model[364] and preparations for future human clinical trial for Chagas' disease are in progress.[365] Structural analysis of the cruzipain−inhibitor complex revealed a covalent Michael adduct with the active site cysteine residue and strong hydrogen bonding interactions in the S1′ subsite.[366] The same compound was also orally effective in a mouse model of malaria.[367,368]

5. Conclusions

Within the past decade, the view about papain-like cysteine proteases has shifted from a small number of house-keeping enzymes of little if any diagnostic and therapeutic value to a large protease family of highly diversified and specific functions. The number of known human cathepsins quadrupled within this time period to a total number of 11. The identification of cell-type and tissue specific cathepsins allowed the discovery of specific functions in tissue remodeling and antigen presentation. The generation of cathepsin-deficient mouse strains revealed novel and specific functions also among ubiquitously expressed proteases such as cathepsins L and B. For example, cathepsin L is required in such diverse physiological processes such as normal development of hair follicles and skin epidermis,[369,370] for cardiac morphology and function,[371] as well as for the positive T-cell selection in thymus.[158,372] On the other hand, cathepsin B appears to be necessary for trypsinogen activation in the pancreas[373] and in the regulation of apoptosis of hepatocytes.[374,375] Cathepsin K-deficient mice confirmed the malfunction of osteoclastic bone resorption[93] initially observed in the human autosomal recessive bone sclerosing disorder, pycnodysostosis, the human form of cathepsin K deficiency.[77] Cathepsin S-deficient mice supported the role of this protease in antigen presentation but indicated at the same time that other related cathepsins may replace this activity in a cell-type specific manner.[154,155] Finally, cathepsin C-deficient mice revealed the essential role of cathepsin C in granzyme A and B activation. It is expected that null mice for the remaining cathepsins (cathepsins H, F, O, W, and X) will follow suite in nearby future and reveal specific functions of these papain-like cysteine proteases. The elucidation of the physiological functions of human cathepsins has opened novel therapeutic windows to treat major human systemic ailments such as excessive bone and cartilage loss in osteoporosis and arthritis or immunologically related diseases such as asthma. Major pharmaceutical efforts are presently underway to design and evaluate highly selective cathepsin K and S inhibitors for the treatment of osteoporosis, rheumatoid arthritis, and asthma. It remains to be seen whether these inhibitors will fulfill the expectations in terms of efficacy and selectivity. A major problem in protease inhibitor design is sufficient selectivity in the light of our still limited knowledge about the specificity of all human cathepsins and their physiological functions.

Equally important is the expanding knowledge about papain-like cysteine proteases expressed in major human and domestic animal disease-causing parasites. Parasite-induced diseases affect more than half of humankind and for various socioeconomic reasons have not obtained the attention of the major life-style related diseases in the western world. Cysteine proteases in those parasites have been demonstrated to be essential for the life cycles and the virulence of the parasites. Very promising preliminary data with cysteine protease inhibitors indicate that the inhibition of papain-like proteases might be of highly beneficial value for the treatment of pandemic diseases such as malaria, Chagas' and sleeping disease and various other parasitic infections.

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