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Review

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Cathepsin C: structure, function, and pharmacological targeting

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Abstract

Cathepsin C is a papain-like cysteine peptidase known primarily for its involvement in the activation of serine peptidases in neutrophils and other immune cells. Its critical role in this process qualifies cathepsin C as a target for the treatment of inflammatory diseases, and its most advanced inhibitor, brensocatib (Insmed), is currently in phase 3 clinical trials for the treatment of non-cystic fibrosis bronchiectasis. Beyond neutrophils, its importance is highlighted by loss-of-function mutations that cause the recessively inherited Papillon-Lefèvre syndrome. At the molecular level, cathepsin C has several structural and functional features that set it apart from other members of the family and enable its selective targeting. It possesses dipeptidyl-peptidase activity (its other common name is dipeptidyl-peptidase I) due to the presence of an additional exclusion domain that also controls its stepwise tetramerization during maturation. In this review article, we summarize the current state of the art regarding the biochemical properties of cathepsin C, its physiological and pathological roles in neutrophils and beyond, and recent advances in the development and evaluation of cathepsin C inhibitors.

Keywords: Dipeptidyl-peptidase I, DPPI, cysteine cathepsin, bronchiectasis, brensocatib

INTRODUCTION

Cathepsin C (also known as dipeptidyl-peptidase I, EC 3.4.14.1) was among the first peptidases identified in

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animal tissues[1]. It belongs to the papain-like family of cysteine peptidases (PLPs), which is classified as family C1, clan CA in the MEROPS database of peptidases, inhibitors, and substrates[2]. It is found in animals and several other eukaryote lineages[3,4]. In humans, it is encoded by the *CATC* gene located at chromosomal location 11q14.2. It is ubiquitously expressed in humans and other mammals[5] and is a unique peptidase both in terms of its biochemical properties and its specific physiological and pathological functions[3]. It plays a pivotal role in the activation of effector serine peptidases in immune cells such as neutrophils, cytotoxic T lymphocytes, and others. This led to the establishment of cathepsin C as a promising target for the treatment of inflammatory diseases that are characterized by excessive activation of cells of the immune system[6]. Numerous more or less successful inhibitors of cathepsin C have been synthesized and evaluated to date[6]. The most advanced inhibitor, brensocatib (Insmed, Inc, USA), is currently undergoing a phase 3 clinical trial for the treatment of non-cystic fibrosis bronchiectasis[7]. Furthermore, cathepsin C is well-known for the fact that mutations in the *CATC* gene cause the recessive hereditary diseases called Papillon-Lefèvre syndrome and Haim-Munk syndrome[8-10], highlighting the systemic effects of cathepsin C deficiency. Symptoms include early periodontitis, pathological thickening of the skin on the palms and feet, and increased susceptibility to infection[11]. Apart from these, many other (patho)physiological functions have been attributed to cathepsin C in recent decades.

In this review, we summarize the current state of the art on the structural and functional properties of cathepsin C, its processing and trafficking in the cell, and its roles in human physiology and pathology. As this review is part of the special issue "Neutrophil Serine Proteases in Rare Diseases", the primary focus is on its roles in the immune system, particularly in neutrophils. In the end, we also provide an overview of current and emerging strategies for the pharmacological targeting of cathepsin C in human disease.

STRUCTURE AND FUNCTION OF CATHEPSIN C

Many expert reviews on the structural and functional properties of PLPs in general are available, e.g., references[3,12], and the reader is advised to refer to these for more in-depth information on this topic. Herein, we are focusing on cathepsin C and its specific structural and functional properties. Like other members of the PLP family, cathepsin C is synthesized as an inactive precursor called procathepsin C. Its active site contains a Cys-His catalytic dyad that forms a thiolate-imidazolium ion pair (residues Cys234 and His381 according to procathepsin C numbering). What distinguishes it from other PLPs is the additional exclusion domain, the fold of which is similar to that of metalloprotease inhibitors[13]. The exclusion domain is located at the N-terminus of procathepsin C and is separated from the catalytic peptidase domain by the propeptide [Figure 1A]. Upon activation, the propeptide (residues Ala111 through His206) is removed, leaving the exclusion domain non-covalently bound to the peptidase domain, which in turn is cleaved into a heavy chain and a light chain by cleavage between Arg370 and Asp371[13]. As its name suggests, the crucial functional role of the exclusion domain is that it sterically hinders access to the active site beyond site S2 [Figure 1B], making cathepsin C a dipeptidyl-peptidase, i.e., an exopeptidase that cleaves dipeptides of the N-termini of its substrates. Moreover, the side chain of residue Asp1 interacts with the N-terminus of the substrate and stabilizes its binding[13]. The selectivity of the S2 binding site, which is the primary specificity determinant of PLPs, is dominated by two negative charges, namely the aforementioned side chain of residue Asp1 at the entrance of the pocket and a chloride ion at the bottom of a deep hydrophobic pocket [Figure 1C]. The latter is required for enzyme activity and is another distinguishing feature of cathepsin C that sets it apart from other PLPs[13,14]. In our recent evolutionary analysis of cathepsin C, we found that residue Asp1 is strictly conserved in all cathepsin C enzymes throughout the phylogenetic tree, suggesting that dipeptidyl-peptidase activity is the conserved enzymatic activity of these enzymes in all species[4]. The substrate binding sites S1, S1', and S2' do not have any special features compared to other members of the family[15]. Cathepsin C has a relatively broad substrate specificity[16-19] and usually acts by the stepwise

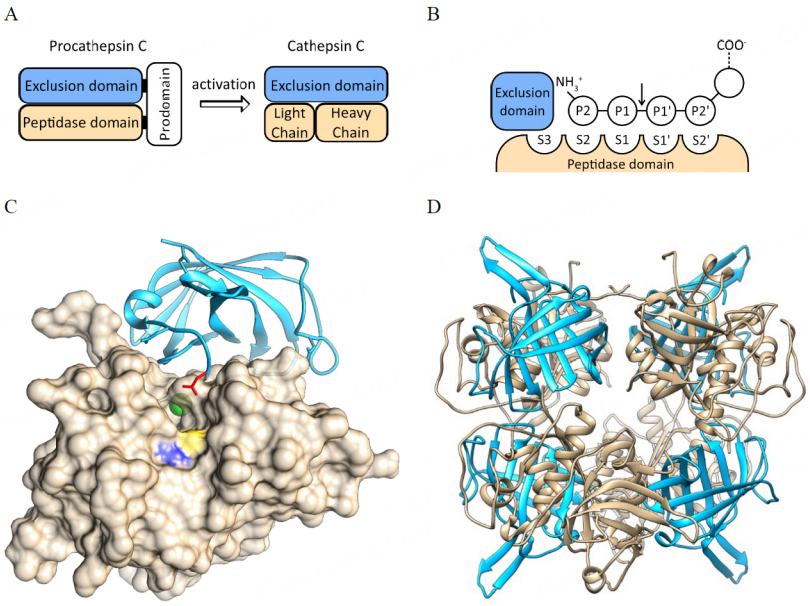


Figure 1. Structural characteristics of cathepsin C. (A) Schematic representation of the (pro)cathepsin C structure; (B) Schematic representation of the active site of papain-like peptidases adapted for cathepsin C. The substrate binding sites on the enzyme are labeled S3 through S2'. The substrate is presented with white circles corresponding to individual residues and residues P2 through P2' are labelled. The arrow denotes the scissile bond; (C) Three-dimensional structure of a cathepsin C subunit. The peptidase domain is shown in molecular surface representation and the exclusion domain in cartoon representation. Active site residues Cys234 and His381 are colored yellow and blue, respectively, Asp1 is shown as red sticks, and the chloride ion cofactor as a green sphere; (D) The cathepsin C tetramer shown in cartoon representation. Exclusion domains are colored blue and peptidase domains are colored tan, respectively. Coordinates were retrieved from the Protein Data Bank under accession code 1K3B.

removal of dipeptides from the N-terminus of the protein substrate. It stops only in three cases: when the N-terminal amino group is blocked, when a proline residue is adjacent to the cleavage site (P1 or P1' position), or when the N-terminal residues are lysine or arginine. Indeed, derivatives of positively charged and aromatic amino acids have been found to competitively inhibit cathepsin C[20]. Nevertheless, the enzyme shows specific preferences for certain residues at positions P1 and P2[16-18] and at positions P1' and P2'[19]. Due to its activity, most substrates used to measure cathepsin C activity *in vitro* are synthetic dipeptide derivatives. The first reported substrates were Gly-Phe-p-nitroanilide (Gly-Phe-pNA) and Gly-Phe-β-naphthylamide (Gly-Phe- βNA)[15]. Their 7-amido-4-methylcoumarin analog (Gly-Phe-AMC) is the most commonly used fluorogenic substrate in the published literature, although better substrates containing natural and unnatural amino acids, such as L-norleucine, L-4-benzoylphenylalanine or L-glutamic acid benzyl ester, have been identified by library screening[16-18]. In addition, highly specific internally quenched substrates have been developed for the detection of cathepsin C activity in biological samples[19]. Like most PLPs, cathepsin C shows optimal activity under slightly acidic pH conditions[21,22]. Unlike others, it also requires chloride (or other halides) ions for optimal activity below pH 7[14,23]. At neutral and basic pH, cathepsin C also exhibits transferase activity[24,25], but similar activity has been observed with other related and unrelated peptidases, e.g., papain[26]. While most cysteine cathepsins are monomers, mature human cathepsin C is a homotetramer organized as a dimer of dimers[13,22] [Figure 1D]. The exclusion domain plays an indispensable role in tetramer formation, as each copy interacts with the peptidase domains of two adjacent subunits. In the tetramer, the active sites of all subunits are fully exposed to the solvent, indicating that all four subunits can function simultaneously. Interestingly, distant cathepsin C homologs have been identified in unicellular eukaryotes, e.g., plasmodium, which were shown to be monomers[16]. We have recently conducted a phylogenetic and computational analysis of the evolution of cathepsin C and found that the tetrameric form is likely restricted to animal cathepsin C homologs[4].

PROCESSING AND ACTIVATION OF CATHEPSIN C

As mentioned in the previous section, cathepsin C is synthesized as an inactive single-chain precursor of ~ 60 kDa per subunit. At this stage, the unique exclusion domain presumably functions as an intramolecular chaperone that aids in the folding of the protein[27]. Evidence shows that human procathepsin C is a homodimer[28,29]. It contains four N-glycosylation sites, one in the heavy chain region of the peptidase domain (Asn252) and three in the exclusion domain (Asn5, Asn29, and Asn95), all of which are retained in the mature form of the enzyme. Proper N-glycosylation is critical for the proper processing and transport of procathepsin C[30], as well as for the assembly of the oligomeric structure, since recombinant non-glycosylated human cathepsin C produced in *E. coli* is a monomer[31]. There is also some interspecies variability in the number of N-glycosylation sites between mammals[4] and in the oligomeric states of cathepsin C orthologs, as rat cathepsin C was reported to be a dimer[32].

Procathepsin C is transported from the endoplasmic reticulum to lysosomes via the mannose-6-phosphate pathway[33,34]. Unlike other cysteine cathepsins, procathepsin C is incapable of autocatalytic processing and must be activated by other lysosomal peptidases. Cathepsins L and S were identified as the first cathepsin C activating peptidases[28]. The maturation of procathepsin C is a multistep process that involves the removal of the internal propeptide segment and the cleavage of the catalytic domain into a heavy chain and a light chain. Full processing is required to obtain the final active conformation[28]. However, it has been shown that cathepsins L and S are not required for the activation of procathepsin C in mice[35]. Similarly, a study on human neutrophil progenitor cell lines PLB-985 and HL60 revealed that complete inhibition of CatS was not sufficient to completely block the activation of procathepsin C, suggesting that other peptidases are involved in this process[36]. Recently, cathepsins F, K, and V were shown to activate procathepsin C *in vitro* via the same intermediate species as cathepsins L and S[29]. These results suggest that the maturation of procathepsin C is a redundant process that can be carried out *in vivo* by different peptidases in tissue-specific patterns.

The purpose of the tetrameric structure of cathepsin C, in contrast to the monomeric forms of other related peptidases, has remained largely unexplained. Olsen *et al.* suggested that the tetrameric structure merely stabilizes the interaction of the exclusion domain with the peptidase domain, thus maintaining the dipeptidyl-peptidase activity[37]. An early report also indicated that cathepsin C is an allosteric enzyme that exhibits pH- and cofactor-dependent cooperative effects[23]. However, these results were later disputed by others[14]. A partial inhibitor of cathepsin C, i.e., one that enables the enzyme to retain part of its activity in the enzyme/inhibitor complex, has been described that could presumably act via allosteric mechanisms at the level of individual subunits[31], a definitive answer to the question of cooperativity between subunits in cathepsin C is still pending.

The non-covalent interactions of the exclusion domain with the peptidase domain and between subunits are strong, and the tetramer does not dissociate in the presence of 2 M guanidinium chloride, but with further increased concentration, the oligomer is completely destroyed[38]. The recombinant peptidase domain alone has been shown to have similar endoproteolytic activity to other papain-like peptidases[39]. In addition, canine and rabbit cathepsins C have been shown to cleave substrates typical of PLP endopeptidases under certain conditions[40,41], suggesting that the exclusion domain can be removed.

PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF CATHEPSIN C

As pointed out in the introduction, a major physiological role of cathepsin C is the activation of latent zymogens of effector serine peptidases in immune cells. These include four neutrophil serine peptidases (NSPs),i.e., neutrophil elastase, proteinase 3, cathepsin G[42], and the recently discovered neutrophil serine

protease 4[43], as well as granzymes in cytotoxic T lymphocytes, and chymases in mast cells. All belong to the chymotrypsin-like family, classified as clan PA, family S1, subfamily S1A in the MEROPS database of peptidases[2]. All are synthesized as preproenzymes and processed to inactive zymogens upon completion of synthesis and ER import. The zymogens contain an N-terminal propeptide of two amino acid residues and are converted to their active forms via a single cleavage by cathepsin C which removes the propeptide[44-46]. This causes a conformational change that results in the formation of a catalytically competent active site. The underlying molecular mechanism is well conserved in chymotrypsin-like peptidases[47] and was also studied at the molecular level for the activation of prochymase[48].

NSPs are synthesized at the promyelocyte stage. Proper timing of the N-terminal processing is essential for their activity and optimal storage in azurophil granules. Activation normally occurs after the sorting of zymogens to pregranule/granule compartments[49]. Garwicz *et al.* have shown that while the N-terminal propeptide is not strictly necessary for the sorting of neutrophil cathepsin G into the granules, premature activation of the zymogen is deleterious[50]. Similarly, abnormal processing in the absence of DPPI has been shown for granzyme A, resulting in products cleaved at alternative locations by unknown peptidases[46]. NSPs can be either stored in an active form in the granules or secreted from the cell as zymogens in varying proportions, depending on the individual peptidase[51]. Activated neutrophils secrete stored, active NSPs as soluble molecules or bound to chromatin in the form of neutrophil extracellular traps[52]. Once released into the extracellular environment, these pro-inflammatory peptidases can degrade various extracellular matrix components, resulting in tissue damage and chronic inflammation[53].

Some tissue studies have provided evidence that mast cells are the predominant cathepsin C-expressing cells in the non-inflamed airways of dogs[40] and mouse skin[54]. Studies in cultured cells suggested that some cathepsin C is packaged and secreted in serine protein-rich mature granules[55]. Whether cathepsin C, which is released into the extracellular environment rich in cystatins and other potential inhibitors, can also act outside the cell by cleaving extracellular proteins remains unclear, although some studies suggest it is capable of doing so[40,41]. The repertoire of mast-cell peptidases activated by cathepsin C includes cathepsin G and chymase[56-58]. Mast cells from cathepsin C-deficient mice express normal amounts of chymase, but it has the form of an unprocessed proenzyme[45]. The involvement of cathepsin C in the activation of tryptases is less clear[45,59,60]. Recent studies suggest that cathepsin C is sufficient, but not necessary, for the complete maturation of β-tryptases in human mast cells, and that one or more other cysteine cathepsins may take over this role[60]. Experiments in animal models support a role for cathepsin C in tryptase activation, as tryptase activity is reduced but not absent in cathepsin C-deficient mice[45]. In addition, studies in animal models indicate that cathepsin C in mast cells increases the likelihood of fatal sepsis, suggesting a role for cathepsin C in regulating interleukin-6 levels by regulating the production of tryptase and other interleukin-6-degrading peptidases[61]. Similarly, granzymes in cytotoxic T lymphocytes contain N-terminal pro-dipeptides (usually Gly-Glu or Glu-Glu)[62,63]. In these cells, cathepsin C is present in secretory granules[64], and several studies have shown that cathepsin C can activate these peptidases *in vitro*[65-67].

Roles in immunity other than activation of serine peptidases have also been described for cathepsin C. Evidence points towards its regulatory role in macrophage polarization into the M1 phenotype via an interplay with tumor necrosis factor α (TNFα), focal adhesion kinase (FAK) and the p38/mitogen-activated protein kinase (MAPK) pathway[68,69]. Similarly, cathepsin C was shown to promote microglia M1 polarization in the brain[70].

The crucial role of cathepsin C in the activation of peptidases in immune cells suggests that its activity is also an important factor in the development of pathological conditions associated with excessive activity of

these cells and their effector peptidases. These pathologies include various inflammatory and autoimmune diseases such as arthritis[71,72], asthma[40], abdominal aortic aneurysm[73], cystic fibrosis[36], pancreatitis[74], neuroinflammation[70,75] inflammatory lung diseases[36] including bronchiectasis[76], and indirectly chronic obstructive pulmonary disease[77]. Inhibition of cathepsin C activity in bone marrow was shown to be an effective method of silencing neutrophil serine peptidases, underscoring the importance of cathepsin C as a target for the treatment of these diseases[78], as discussed in more detail in the next section.

As expected for a ubiquitously expressed protein, physiological and pathological roles and substrates outside of the immune system have also been described for cathepsin C. The best known pathological examples are mutations in the *CATC* gene that cause the recessive hereditary diseases called Papillon-Lefèvre syndrome (PLS) and Haim-Munk syndrome (HMS)[8-10]. Symptoms of both diseases include early periodontitis, pathological thickening of the skin on the palms and feet, and increased susceptibility to infection, again highlighting the important role of cathepsin C in the immune system[11]. Neutrophil function is impaired but not completely abolished[79]. At the molecular level, mutations can result in truncated cathepsin C[9] or point mutations that cause loss of function[8,9]. At the cellular level, autophagy is impaired, and treatment of PLS patients with recombinant cathepsin C has been proposed as an approach to restore autophagic function[80].

Several studies pointed to the roles of cathepsin C in the systemic regulation of metabolism. The earliest study showed that cathepsin C inactivates the peptide hormone glucagon by sequential removal of dipeptides from the N-terminus[21]. Cathepsin C, in conjunction with plasma glutamate carboxypeptidase, was also shown to be involved in the extracellular processing of thyroglobulin, which is coupled with the release of the stress hormone thyroxin from the thyroid gland[81]. Furthermore, it has been shown to participate in lysosomal degradation of the digestive peptide hormone cholecystokinin together with tripeptidyl peptidase I (TPP-1)[82]. Due to their overlapping N-terminal exopeptidase activity, it was first suggested that cathepsin C may compensate for lack of TPP-1 activity and be used to alleviate the deleterious effects of TPP-1 loss-of-function mutations, which cause the lethal neurodegenerative lysosomal storage disorder classical late-infantile neuronal ceroid lipofuscinosis (CLN2)[82,83]. Unfortunately, studies using mouse models of the disease have invalidated this approach, as cathepsin C did not functionally compensate for the loss of TPP-I activity in the brain[84].

There is also increasing evidence that cathepsin C contributes to the progression of various cancers such as squamous cell carcinoma[54], hepatocellular carcinoma[85], gastric cancer[86], colorectal cancer[87], breast cancer[88], and so on. In these cancers, pathological cathepsin C activity originates either from tumor stromal cells[54] or directly from tumor cells[85-88], and contributes to tumorigenesis via diverse cellular and molecular mechanisms. In squamous cell carcinoma, increased activity of cathepsin C was observed in fibroblasts and bone marrow-derived cells of the tumor stroma, which promoted carcinogenesis[54]. In hepatocellular carcinoma, cathepsin C/TNFα/p38 MAPK interplay was observed similar to that contributing to macrophage M1 polarization[85], whereas in gastric and colorectal cancer, cathepsin C was associated with dysregulation of autophagy[86,87]. In breast cancer, cathepsin C was found to be secreted from tumor cells and to promote neutrophil recruitment during lung metastasis[88]. A detailed expert review on the indirect and direct roles of cathepsin C in cancer has been published recently[89].

In vitro, cathepsin C also plays a critical role in the permeabilization of the lysosomal membrane triggered by the lysosomotropic detergent L-leucyl-leucine methyl ester (LLOMe), resulting in the release of lysosomal hydrolases into the cytosol that ultimately triggers caspase-dependent apoptotic cell death. Cathepsin C has been proposed to mediate lysosomal membrane permeabilization by catalyzing the

polymerization of LLOMe accumulated in lysosomes into its membranolytic polymeric form[90].

In vivo, cathepsin C activity is regulated by endogenous proteinaceous inhibitors. Like other PLP, it is amendable to inhibition by inhibitors from the cystatin family[91,92]. Of note, it is also inhibited by cystatin F[93], which is expressed only in immune cells[94]. Interestingly, inhibition depends on the proteolytic processing of cystatin F to remove its N-terminal part, which cannot be accommodated into the active site of cathepsin C[93]. Recently, it was found that Glu-Lys and Gly-Glu dipeptides derived from the pro-region of granzymes A and B, respectively, inhibit the transferase activity of cathepsin C with IC_{50} values < 20 mM at pH 7.4 and peptidase activity at pH 5.5 with K_i values of 20 mM and 2.5 mM for Glu-Lys and Gly-Glu, respectively[95]. Although this finding is unlikely to be considered as a strategy for pharmacological targeting of cathepsin C, it is one of the few documented examples of product inhibition in this family of peptidases and provides additional insight into the diversity of cathepsin C regulation *in vivo*.

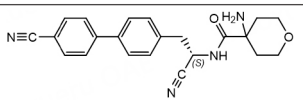
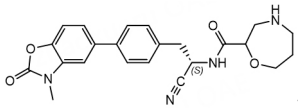
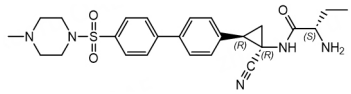
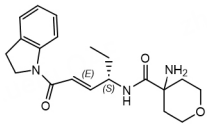
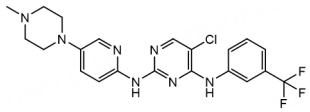
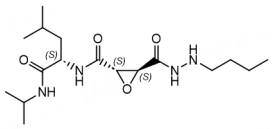
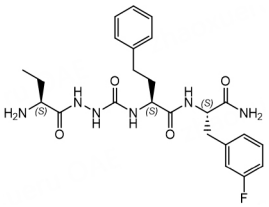
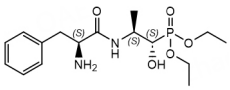
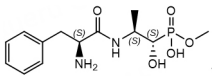
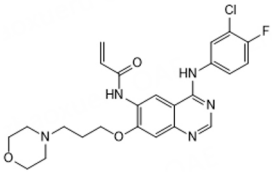
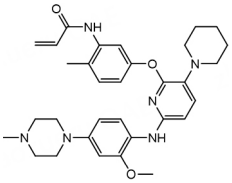
PHARMACOLOGICAL INHIBITION OF CATHEPSIN C

Because of its critical roles in the activation of neutrophil and other serine peptidases, cathepsin C is considered a promising target for the treatment of diseases associated with overactivity of cells producing and secreting these peptidases, such as chronic inflammatory diseases, autoimmune diseases, and so on. Cathepsin C is by no means an easy target in these diseases, as successful anti-cathepsin C therapies must achieve a sustained inhibitory effect directly in the bone marrow to prevent the maturation of NSPs and other serine peptidase zymogens in maturing immune cells[78,96]. Cathepsin C is also an emerging target for the treatment of various cancers[89]. An expert review dedicated specifically to the pharmacological targeting of cathepsin C has been published recently[6]. Here, we shall briefly summarize the current state-of-the-art in the inhibition of cathepsin C.

Most cathepsin C inhibitors are peptide-based and form covalent bonds to the catalytic residue Cys234 using reactive diazomethyl-ketone, nitrile, semicarbazide, or vinyl-sulfone groups (i.e., "warheads"). Some of the most advanced cathepsin C inhibitors to date are collected in Table 1. One of the earliest reported cathepsin C inhibitors was Gly-Phe-CHN_2[97], a substrate analog from a series of diazomethyl-ketone warhead-containing irreversible inhibitors (inactivators) of cysteine peptidases[98]. The high reactivity of the diazomethyl ketone group and instability in acidic media limited the use of such probes beyond *in vitro* experiments, as they are metabolically unstable and sometimes have deleterious side effects, while increased metabolic stability of such inhibitors was often accompanied by decreased inhibitory activity[99].

The first cathepsin C inhibitor selected as a candidate for *in vivo* studies was (S)-4-amino-N-(1-cyano-2-(4'-cyano-[1,1'-biphenyl]-4-yl)ethyl)tetrahydro-2H-pyran-4-carboxamide, a reversible inhibitor with a nitrile warhead developed by AstraZeneca (UK) and later named AZD5248[100] [Table 1]. The compound had pIC_{50} values of 9.1 ±0.1 for purified cathepsin C *in vitro* and 8.1 ± 0.1 in a cell-based assay[100]. The crystal structure of AZD5248 bound into the active site of cathepsin C showed that it interacts with the non-primed sites of the enzyme as well as the N-terminal part of the exclusion domain [Figure 2][100]. It effectively inhibited the activation of NSPs in rats[101] but showed aortic binding in quantitative whole-body autoradiography studies, which could lead to potential cardiovascular toxicity. At the molecular level, aortic binding has been associated with cross-reactivity of AZD5248 with aldehydes, resulting in cross-linking with elastin in the aortic wall[102]. Therefore, novel inhibitors based on AZD5248 were prepared to overcome aortic binding, including the most advanced inhibitor to date, AZD7986 ((S)-N-((S)-1-cyano-2-(4-(3-methyl-2-oxo-2,3-dihydrobenzo-[d]oxazol-5-yl)phenyl)ethyl)-1,4-oxazepane-2-carboxamide)[103]. Like AZD5248, AZD7986 is also a highly potent, reversible, and selective inhibitor of cathepsin C with pIC_{50} values of 8.4 for cathepsin C *in vitro* and cell-based assays[103]. It almost completely inhibited the activation of NSPs in primary $CD34^+$

Table 1. Selected synthetic inhibitors of human cathepsin C

Identifier	Structure	Warhead / Type of inhibition	Potency
AZD5248		Nitrile/reversible	pIC_{50} = 9.1 ± 0.1 (*in vitro*) pIC_{50} = 8.1 ± 0.1 (cell-based assay)
AZD7986		Nitrile/reversible	pIC_{50} = 8.4 ± 0.17 (*in vitro*) pIC_{50} = 6.84 ± 0.33 (cell-based assay)
IcatC		Nitrile/reversible	IC_{50} = 15 ± 1 nM
GSK2793660		α,β-unsaturated amide/irreversible	IC_{50} = 0.43-1 nM
Compound 54 in ref.[115]		Pyridine/reversible	IC_{50} = 57.4 ± 0.7 nM
Compound 11 in ref.[117]		Epoxide/irreversible	k_{inact} = (5.6 ± 1.7) × 10^4 M^{-1} s^{-1}
Compound 8 in ref.[121]		Semicarbazide/reversible	IC_{50} = 31 ± 3 nM K_i = 45 ± 2 nM
Compound 3c in ref.[122]		Phosphonate (non-covalent)/reversible	K_i = 23 ± 12 nM
Compound 4a in ref.[122]		Phosphonate (non-covalent)/reversible	K_i = 51 ± 16 nM
Canertinib		Acrylamide/irreversible	IC_{50} = 0.12 μM
Compound 22 in ref.[125]		Acrylamide/irreversible	IC_{50} = 17.3 ± 0.9 nM (*in vitro*) IC_{50} = 1-5 nM (cell-based assay)

neutrophil progenitor cells from human bone marrow with a pIC_{50} value of 6.84 ± 0.33 and had favorable predicted plasma stability and clearance rate[103].

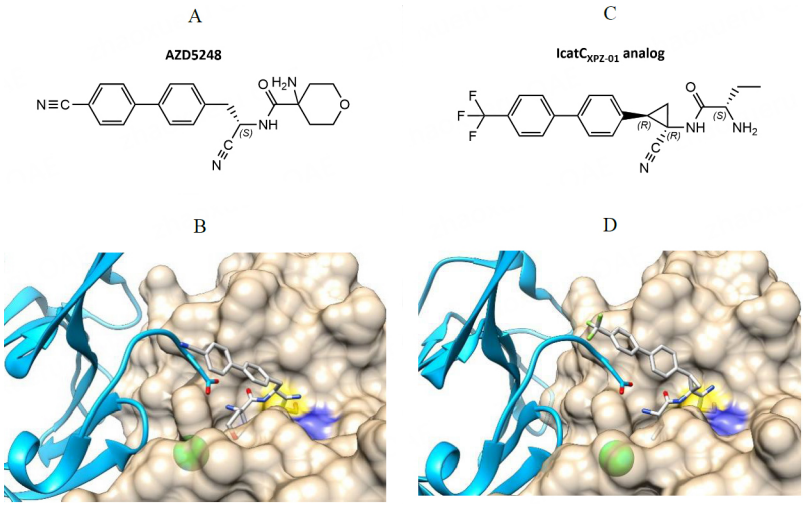


Figure 2. Reversible nitrile warhead-containing inhibitors of cathepsin C. (A) Chemical structure of compounds AZD5258 developed by AstraZeneca; (B) Crystal structure of AZDB5248 bound into the active site of cathepsin C. (PDB accession code 4CDE); (C) Chemical structure of the IcatC$_{XPZ-01}$ analog used for crystallization; (D) Crystal structure of IcatC$_{XPZ-01}$ analog bound into the active site of cathepsin C (PDB accession code 6IC6). Chemical structures were drawn with ChemDraw software. The crystal structures in panels B and D were drawn with UCSF Chimera software[113].

AZD7986 was the first nitrile-based cathepsin C inhibitor to reach clinical trials[104]. In 2016, Insmed, Inc (USA) announced a license agreement with AstraZeneca for exclusive worldwide rights to AZD7986. The compound was renamed INS1007 (trade name brensocatib) and was used in a trial study for the treatment of non-cystic fibrosis bronchiectasis, a persistent dilatation of the airways associated with neutrophil-mediated inflammation[76]. In the 24-week phase 2 study, which ended in 2020, patients treated with brensocatib showed reduced NSP activity, which was associated with the clinical effect of bronchiectasis[105]. These results confirmed the efficacy of brensocatib in the treatment of chronic neutrophil-related inflammatory diseases in the lung. A phase 3 clinical trial is currently underway[7]. Neutrophils and their peptidases are also associated with the severity of COVID-19[106,107]. Therefore, brensocatib has also been evaluated for its beneficial effects in hospitalized patients with COVID-19. Unfortunately, brensocatib did not improve the clinical condition of COVID-19 patients[108].

Another promising nitrile-based inhibitor of cathepsin C is the compound IcatC$_{XPZ-01}$ ((S)-2-amino-N-((1R,2R)-1-cyano-2-(4'-(4-methylpiperazin-1-ylsulfonyl)biphenyl-4-yl)cyclopropyl)butanamide) [Table 1]. IcatC$_{XPZ-01}$ is a reversible, potent, and cell-permeable inhibitor with an IC$_{50}$ value of 15 ± 1 nM and excellent selectivity for cathepsin C[78]. The crystal structure of one of its derivatives bound to the active site of cathepsin C revealed a binding pose analogous to that of AZD5248 [Figure 2][109]. The compound successfully inhibited the activation of NSPs in bone marrow in both cell-based assays and primate experiments[78]. In mice, sufficient concentrations of IcatC$_{XPZ-01}$ were accumulated in the bone marrow to inhibit cathepsin C, and subcutaneous administration of the inhibitor showed significant anti-arthritic activity in an anti-collagen-induced rheumatoid arthritis mouse model[109]. Moreover, preoperative treatment of mice prior to lung transplantation improved early graft function and decreased active NSP levels in the graft, indicating a novel potential use of cathepsin C inhibitors[110]. Despite its promising potential, there is currently no information on its further exploration as a candidate for human clinical trials.

In addition to AZD7986, two other cathepsin C inhibitors have entered clinical trials. Compound GSK2793660 [Table 1] was developed by GlaxoSmithKline (UK) and is a dipeptide-based irreversible inhibitor with an α,β-unsaturated amide-reactive group. Unfortunately, clinical trials were discontinued after several volunteers in a phase 1 study showed symptoms of epidermal desquamation of the palms and soles after repeated administration of the inhibitor, which bore some resemblance to PLS patients. In addition, no significant reduction in NSP activity was observed[111]. The third compound, BI1291583 (undisclosed structure), developed by Boehringer Ingelheim (Germany), has just started a phase 2 trial for the treatment of bronchiectasis (NCT05238675) after successfully completing phase 1[112] and is expected to be completed in Q1 of 2024.

Based on the successes and failures of these inhibitors, novel non-peptidic, non-covalent inhibitors of cathepsin C are emerging in recent years[114]. Chen *et al.* synthesized and characterized a series of inhibitors based on a pyridine scaffold. The best compound had a reported IC_{50} value of 57.4 ± 0.7 nM and was selective for cathepsin C *in vitro*[115] [Table 1]. Its administration also reduced NSP activation in rat bone marrow and showed anti-inflammatory activity in a rat model of COPD[115]. The compound was recently further optimized to improve its pharmacokinetic properties[116]. Further research will shed light on the efficacy and safety of these compounds.

In addition, a substantial number of cathepsin C inhibitors have been reported that have provided only *in vitro* data and no *in vivo* follow-up studies to date. Using a structure-based approach, Radzey *et al.* used E-64c hydrazide as a lead structure for the development of irreversible cathepsin C inhibitors[117]. The best resulting inhibitor, (2S,3S)-3-(2-butylhydrazine carbonyl)-N-((S)-1-(isopentylamino)-4-methyl-1-oxopentan-2-yl)oxirane-2-carboxamide [Table 1], exhibits significantly improved potency (k_{inact} = (5.6 ± 0.17) × 10^4 M^{-1} s^{-1}) compared to E-64c-hydrazide (k_{inact} = 140 ± 5 M^{-1} s^{-1}). It also reacts more rapidly with cathepsin C than with cathepsin L, which is the opposite of E-64c and its hydrazide, which have a strong preference for cathepsin L[117]. The authors therefore proposed this compound as a starting point for the development of optimized inhibitors that bind to the S1'-S2' sites of cathepsin C[117].

Azapeptides, i.e., peptide analogs in which one or more amino acids have been replaced by a semicarbazide group, have been reported as inhibitors of hepatitis C virus NS3 peptidase[118], human rhinovirus 3C peptidase[119], and several papain-like cysteine peptidases[120]. Azapeptides with weak leaving groups cannot acylate the enzyme and therefore show competitive inhibition, whereas azapeptides with reactive leaving groups form carbamoyl-enzyme complexes that are more stable than normal acyl enzymes. For cathepsin C, one of the best inhibitors from this class was 1-(2S-2-aminobutanoyl)-4-{2S-N-[2S-3-(m-fluoro-phenyl)propan-2-yl-amide]-4-phenylbutan-2-yl-amide}semi- carbazide [Table 1] with an IC_{50} value of 31 ± 3 nM and a K_i value of 45 ± 2 nM. The compound acted as a reversible, competitive inhibitor and was selective for cathepsin C. It was not cytotoxic to HepG2 cells and showed about 50% inhibition of intracellular cathepsin C activity in this cell line[121].

Drag *et al.* described a series of phosphonate dipeptide analogs as non-covalent inhibitors of cathepsin C [122]. The most potent compounds, diethyl 2-(L-phenylalanyl) amino-1-hydroxymethane phosphonate and monomethyl 2-(L-phenylalanyl) amino-1-hydroxyethane phosphonate [Table 1], inhibited cathepsin C with K_i values in the nanomolar range (23 ± 12 nM and 51 ± 16 nM, respectively). Unfortunately, these compounds exhibited low selectivity for cathepsin C, as they have also been shown to be potent inhibitors of other cysteine peptidases such as papain, cathepsin B, and cathepsin K[122]. Nevertheless, the phosphonate dipeptide analogs identified in this study could serve as lead compounds for the development of more specific inhibitors of cathepsin C and/or other cysteine cathepsins. Similarly, a group of tripeptide

aminophosphonates were recently identified as weak or moderate inhibitors of cathepsin C[123], further highlighting the potential of the phosphonate group as transition state analogs.

Moreover, two known epidermal growth factor receptor (EGFR) inhibitors containing Cys-reactive acrylamide warheads were identified as cathepsin C inhibitors by activity-based protein profiling, highlighting the potential of drug repurposing for the targeting of cathepsin C. In 2016, Canertinib, a former drug candidate developed by Pfizer (USA) for the treatment of cancer, was identified as a potent cathepsin C inhibitor with an IC_{50} value of 0.12 μM [Table 1]. Based on the structure of Canertinib, novel molecular probes with additional tags and/or click chemistry-compatible reactive groups were synthesized, providing the potential basis for further development of selective cathepsin C inhibitors[124]. Later, the same group reported that the EGFR inhibitor WZ4002 inhibits cathepsin C with an IC_{50} value in the micromolar range[125]. A new series of inhibitors developed on this scaffold led to the identification of a highly potent and selective inhibitor, N-(5-((6-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)- 3-(piperidin-1-yl) pyridin-2-yl)oxy)-2-methylphenyl)acrylamide (compound 22), [Table 1]. *In vitro*, the compound showed concentration-dependent inhibition of cathepsin C activity in both THP1 and U937 cell-based assays, with IC_{50} values of 2 and 3 nM, respectively. It also showed good metabolic stability and oral bioavailability and resulted in efficient inhibition of downstream neutrophil serine peptidases in both bone marrow and blood of mice[125].

Naturally occurring compounds are a rich source of pharmacologically active molecules and inhibition of cathepsin C by such compounds has also been investigated to some extent. A recent study has shown that polyglucoside from the vine *Triperygium wilfordii* inhibited cathepsin C activity in the serum, synovial fluid, and tissues in a rat model of collagen-induced arthritis, highlighting the possibility that the suppression of rheumatoid arthritis traditionally associated with *Tripergyium wilfordii* polyglucoside may be related to the inhibition of cathepsin C and downstream serine peptidases[126]. In addition, we found that caffeic acid and its derivatives inhibit cysteine cathepsins, including cathepsin C[31,127], *in vitro*. Since polyphenolic compounds such as caffeic acid have a broad spectrum of pharmacological activity and biological targets, their use for specific targeting of individual protein targets is not feasible. Nevertheless, caffeic acid is considered a promising pharmacophore for the development of specific inhibitors[128]. Alternative methods for targeting cathepsin C are also emerging. We have identified 3'-nitrophthalanilic acid as a partial inhibitor of cathepsins K and C that presumably acts by binding outside of the active site[31,129]. Such allosteric drugs are a popular and promising approach for the targeting of membrane receptors[130] and strategies of targeting sites away from the active site have also been explored in several PLPs, including human cathepsin K[131,132] and plasmodial cathepsin C homologs[133]. However, in the case of human cathepsin C, the development of specific partial inhibitors suitable for use in cell culture and *in vivo* and the evaluation of the efficacy of such an approach are still in their infancy.

CONCLUSION

Although cathepsin C was among the first peptidases to be identified, interest in this peptidase as a drug target has only emerged in the last two decades, primarily leading to facilitated investigation of its physiological and pathological roles. With two inhibitors currently in clinical trials for the treatment of non-cystic fibrosis bronchiectasis, cathepsin C is currently the most important and promising human cysteine cathepsin from a clinical perspective. Considering the recent failures in the field of peptidase targeting, e.g., the cathepsin K inhibitor odanacatib (Merck & Co., USA) for the treatment of osteoporosis which was discontinued after the completion of phase 3 clinical trials[134], the outcomes of these trials may be crucial to (re)establish not only cathepsin C, but cysteine cathepsins and peptidases in general as valid drug targets in human diseases. From the perspective of basic protein science, cathepsin C is proving to be an interesting

model protein for studying the molecular mechanisms and evolution of protein oligomerization[4]. Further research in this direction could, in turn, provide novel strategies for the targeting of this important peptidase.

DECLARATIONS

Authors' contributions

Wrote and revised the manuscript: Stojkovska Docevska M, Novinec M

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Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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