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DISCUSSION

Cysteine Cathepsins and Drug Discovery: Knowns and Unknowns

Andrey A. Zamyatnin, Jr.[1,2,3]

[1]*Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, 119234 Moscow, Russia*
[2]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia*
[3]*Department of Biological Chemistry, Sechenov First Moscow State Medical University, 119991 Moscow, Russia*
e-mail: zamyat@belozersky.msu.ru

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Abstract—Cysteine cathepsins are a group of closely related proteolytic enzymes active at low pH. The most well-studied function of these enzymes is protein degradation within lysosomes. However, accumulating evidence suggests that cysteine cathepsins also function at physiological pH levels in other cellular compartments outside lysosomes, as well as in the extracellular space. Many of these extra-lysosomal functions of cysteine cathepsins are typically associated with pathological processes, contributing to conditions such as oncogenesis and metastasis, neurodegenerative diseases, cardiovascular disorders, and autoimmune and inflammatory processes. Consequently, cysteine cathepsins have been proposed as diagnostic and prognostic molecular markers, as well as pharmacological targets. Notably, the pathological processes involving these enzymes often operate independently of their classical lysosomal functions. This work aims to outline key questions, the answers to which could enhance our understanding of the fundamental mechanisms governing the extra-lysosomal functions of cysteine cathepsins. Addressing these questions is also critical for developing novel therapeutic strategies to treat diseases in which cysteine cathepsins play a pathogenic role.

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The term “cathepsin” was proposed in 1929 by Richard Willstätter and Eugen Bamann [1]. It originates from the Ancient Greek compound word καθέψω (from κατά, meaning “down,” and hépsō, meaning “to boil”), which can be translated as “to boil down” or “to digest”. Thus, the term reflects the most well-known function of these proteases: as enzymes responsible for the breakdown of proteins in lysosomes. The proteolytic apparatus of human lysosomes includes 15 cathepsins. The majority belong to the cysteine protease family (11 enzymes: cathepsins B, C, F, H, K, L, O, S, V, W, and Z [also known as cathepsin X]). Additionally, two enzymes each belong to the serine (cathepsins A and G) and aspartate (cathepsins D and E) protease families [2]. It is noteworthy that all 11 cysteine cathepsins belong to the papain-like C1A subfamily, according to the MEROPS classification [3]. Among human cysteine cathepsins, there are endopeptidases with broad substrate specificity (cathepsins F, K, L, O, S, V, and W), exopeptidases (cathepsins C and Z), as well as enzymes possessing both endo- and exopeptidase activity (cathepsins B and H) [2]. Hence, the optimal conditions for both the autocatalytic activation of cysteine cathepsins and the manifestation of their proteolytic activity are determined mostly by an acidic pH, which is characteristic of the endolysosomal system in cells [2].

Cysteine cathepsins also perform a number of important functions outside lysosomes. For example, cathepsin K is involved in degrading collagen and other components of the bone matrix. To accomplish this, the enzyme is secreted into the resorption lacuna (the zone of contact between an osteoclast and bone), where the local environment is acidified, facilitating the expression of its proteolytic activity [4, 5]. It has also been reported that cathepsins perform functions

in intracellular compartments where physiological pH is maintained. In particular, cathepsin L regulates the degradation of the transcription factor CDP/Cux in the nucleus and is involved in cell proliferation and DNA repair [6, 7]. Cathepsin S cleaves the pro-apoptotic protein Bax in the cytoplasm, thereby participating in the regulation of apoptosis initiation [8]. In the secretory vesicles of pancreatic β-cells, where the pH ranges from 6.5 to 7.0, cathepsin L cleaves proenkephalin and proprotein convertases (PC1/PC2) [9, 10].

The list of physiological substrates for cysteine cathepsins that are cleaved at neutral pH, as discussed above, is relatively short. Nevertheless, it demonstrates the fundamental principle that these enzymes can possess specific functions mediated by their proteolytic activity at physiological pH. This list of substrates could be significantly expanded to include proteins cleaved by cysteine cathepsins in various pathological states. For instance, cathepsin S has been shown to cleave numerous substrates, thereby promoting angiogenesis and tumor growth [11], and is also involved in processing the chemokine CX3CL1 in atherosclerosis [12]. During oncogenesis, E-cadherin is a substrate for cathepsins B, L, S, and V, but not for cathepsin C [13, 14]. Similarly, cathepsin V can cleave N-cadherin and fibronectin [14]. Of particular note is that identical substrates (e.g., collagen, elastin, E-cadherin) are cleaved by various cysteine cathepsins, suggesting a degree of functional redundancy among specific members of this enzyme group [15, 16].

The available data on the involvement of cysteine cathepsins in pathological processes have logically stimulated the development of approaches to use these enzymes and their genes as diagnostic and prognostic molecular markers, as well as therapeutic targets for pharmacological intervention [17, 18]. Initially, the focus was on the development and pharmacological application of enzyme inhibitors. To date, several clinical trials aimed at investigating the effects of cathepsin K, S, C, and B inhibitors have been completed or discontinued (Table 1; see also review [18]). These trials were designed to explore the potential application of inhibitors for treating osteoporosis, inflammatory diseases (including viral and autoimmune conditions), and cancer. However, despite the clearly observed physiological effects – including those causing side effects – exerted by cysteine cathepsin inhibitors, none have been approved as drugs, and their further development (with the exception of a single inhibitor) has been discontinued. This outcome can be attributed to several key factors. For instance, the perception of cathepsins as exclusively lysosomal enzymes still prevails, which has led to insufficient attention being paid to studying their extra-lysosomal functions in disease pathogenesis. Furthermore, the high degree of structural and functional homology among members of the C1A subfamily of cysteine proteases must be considered. This homology results in potential functional redundancy overlap and compensatory mechanisms, complicating the development of specific inhibitors for individual enzymes [2, 16, 22].

Collectively, these issues underscore the necessity for a comprehensive approach to developing therapeutic strategies. Such strategies should target a specific cysteine cathepsin while also accounting for potential off-target effects on other subfamily members. This approach must consider all stages of the biogenesis of these proteolytic enzymes. For example, in addition to the conventional regulation of expression and sorting, cysteine cathepsins undergo a multistep activation process before becoming functional enzymes [2]. Moreover, the determinants governing their activity and substrate specificity remain incompletely understood. These determinants likely differ significantly between the acidic lysosomal milieu and the neutral conditions at physiological pH [23].

The majority of cathepsins (B, H, L, C, X, F, O, and V) are expressed in virtually all tissues. In contrast, cathepsins K, S, and W exhibit a more restricted, tissue-specific distribution; for example, cathepsin K is expressed predominantly in osteoclasts, cathepsin S in immune cells, and cathepsin W in lymphocytes [2]. Furthermore, numerous studies indicate that the expression of certain cysteine cathepsin genes is upregulated during the development of various pathological processes [24-26]. A recent comprehensive study profiling the expression of all 11 human cysteine cathepsins detected their expression in both embryonic tissue-derived cell cultures and cancer cell lines [27]. This study also revealed significant differences in cysteine cathepsin expression between cells of cancerous and embryonic origin. Specifically, in renal carcinoma cells, the expression of cathepsins V, B, L, and S was 3- to 9-fold higher than in embryonic kidney cells, whereas the expression of cathepsins F and X was significantly reduced [27].

The expression of cysteine cathepsins can be regulated at both the transcriptional and post-transcriptional levels [2]. Available data suggest the existence of complex signaling cascades that regulate the transcription of cathepsin genes. For example, it has been shown that increased expression of stefin A – a natural inhibitor of cathepsins – also leads to enhanced expression of cathepsin B, and *vice versa* [28]. However, the mechanisms underlying this compensatory regulation of cathepsin gene expression have not yet been studied in detail. Therefore, the existence of such regulatory mechanisms must be thoroughly investigated and subsequently taken into account when developing agents for the pharmacological targeting of cysteine cathepsins.

Table 1. Cysteine cathepsin inhibitors evaluated in clinical trials

Target (cathepsin)	Inhibitor (name/code)	Clinical trial phase	Condition	Brief description and status	References
K	ONO-5334	phase II	osteoporosis	the inhibitor demonstrated efficacy in reducing bone resorption **development was discontinued (2012)** due to hyperostosis and cardiovascular risks	NCT01384188 NCT00532337 [19]
K	odanacatib (MK-0822)	phase III	osteoporosis	has been shown to reduce the risk of fractures **development was discontinued (2016)** due to an increased risk of stroke and atherosclerosis	NCT00729183 [20, 21]
K	balicatib (AAE581)	phase II	osteoporosis, knee osteoarthritis	**development was discontinued (2007)** due to scleroderma-like skin lesions	NCT00371670
K	MIV-711	phase IIa	knee osteoarthriti	has been shown to reduce bone/cartilage degradation biomarkers but without clinical improvement in symptoms **development was discontinued (2020)**	NCT02705625
S	RO5459072 (RG-7625)	phase I/ Ib/II	– Sjögren's disease – celiac disease – rheumatoid arthritis	– in a study on Sjögren's disease, it demonstrated a reduction in cathepsin S activity but without significant improvement in symptoms (dryness, inflammation) – for celiac disease, it did not meet the primary endpoints (histological improvement) – in rheumatoid arthritis, it did not demonstrate advantages over standard therapy **development was discontinued (2021)**	NCT02679014 NCT02701985 NCT02521610
S	LY3000328	phase I	solid tumors	**development was discontinued (circa 2015)** with no publication of results	NCT01515358
B	VBY-376	phase I	hepatitis C (proposed indication)	a safety study in healthy volunteers was completed in 2009 the results were not published **development was discontinued**, presumably due to a change in company strategy	NCT00557583
C	ADZ7986 (brensocatib; INS1007)	phase III phase II phase II	– bronchiectasis – COVID-19 (severe cases) – chronic obstructive pulmonary disease	– primary endpoint: reduction in the frequency of exacerbations; phase II data demonstrated efficacy (36% reduction) – it was intended to suppress the "cytokine storm" through neutrophil inhibition **development was discontinued (2022)** – a reduction in inflammatory biomarkers ($p < 0.001$) was observed, but with no improvement in lung function **development was discontinued (2020)**	NCT04594369 NCT04817332 NCT03218917

The expression of cysteine cathepsins is also regulated by various epigenetic mechanisms. For instance, the expression of specific cathepsins is modulated by the methylation of CpG islands within their genes' promoter regions [29, 30]. Furthermore, their regulation via long non-coding RNAs [31, 32] and microRNAs [32-35] has been described. However, published studies generally lack a systematic approach to investigating this regulatory landscape. Specifically, the potential activation of compensatory mechanisms mediated by the functional activity of other cysteine cathepsin subfamily members has not been explored.

The intracellular sorting and secretion of cysteine cathepsins through the endoplasmic reticulum and Golgi apparatus, followed by endosome formation, have been described in detail [2]. For some cathepsins, post-translational modifications – including glycosylation – are also known to occur during the sorting process [2]. However, the mechanisms that transport cysteine cathepsins to other intracellular compartments are not well understood. One proposed mechanism suggests that some cathepsin molecules may enter the cytoplasm upon lysosomal membrane permeabilization [36]. Nevertheless, this does not account for the nuclear localization observed for some enzymes in this group [37, 38], as no canonical nuclear localization signals have been identified in their primary structure. Thus, it can be concluded that the sorting mechanisms of cysteine cathepsins – with the exception of their lysosomal and extracellular localization – remain poorly understood and require further investigation.

The expression of a cysteine cathepsin does not necessarily mean that an active enzyme is present in the cell, as a catalytic activation process must occur after translation to produce the active form. The maturation of papain-like cysteine proteases is a multistep process that typically involves the sequential cleavage of the signal peptide and the prodomain. This leads to the release of the active proteolytic domain, which constitutes the mature, active form of the protease [39]. Intriguingly, expressing the proteolytic domain separately from the prodomain in living systems does not yield an active enzyme, suggesting that proteolytic activation involves essential structural rearrangements beyond simple cleavage for the maturation of the active enzyme [40]. Cysteine cathepsins are typically capable of autocatalytic activation under acidic conditions [2, 39], a mechanism that satisfactorily explains the accumulation of their active forms within lysosomes. However, the presence of active cysteine cathepsins in other cellular compartments or the extracellular space may arise from one of three mechanisms: (i) the enzyme is autocatalytically activated within lysosomes and subsequently transported out; (ii) the autocatalytic activation is initiated by additional factor(s) at physiological pH (e.g., it is known that activation can be enhanced by polyanions, particularly glycosaminoglycans [41]); or (iii) their proteolytic activation is mediated by another protease active at physiological pH. Available literature data suggest that all these scenarios are plausible, but the regulatory details and fine-tuning of these processes remain to be elucidated.

A substantial body of data on the enzymatic activity of cysteine cathepsins has been accumulated to date. Overall, the endopeptidase activity of these enzymes is characterized by relatively broad substrate specificity, with the hydrophobic S2 pocket of the active site considered the primary determinant of their substrate preferences [23, 42]. This understanding underpins modern strategies for developing cathepsin inhibitors. It is important to note, however, that most experimental data have been derived from studies conducted in a lysosomal context – that is, under the acidic pH conditions characteristic of these organelles [43]. Concurrently, recent literature indicates that at a neutral pH (representing physiological conditions outside lysosomes), cysteine cathepsins can exhibit heightened substrate specificity, selectively cleaving particular proteins [23]. Nevertheless, the molecular mechanisms governing substrate specificity at physiological pH remain incompletely understood. A detailed understanding of these mechanisms is crucial for developing effective cathepsin inhibitors with defined specificity profiles, including both broad- and narrow-spectrum agents.

In summary, while a reasonably comprehensive understanding of the classical lysosomal functions of cysteine cathepsins has been established, their roles outside the lysosome – under both normal and pathological conditions – remain systemically poorly understood. Consequently, while these enzymes represent well-characterized pharmacological targets for lysosomal pathologies [44], their extralysosomal functions present a major knowledge gap. This gap encompasses the regulation of their expression, maturation, intracellular trafficking, substrate specificity, and other activity-governing parameters. Their partial functional redundancy is a particularly noteworthy aspect. This lack of systemic knowledge explains the limited progress in drug development, which thus far has been confined to enzyme inhibitors. However, it also reveals vast opportunities for future research. Pursuing this agenda will yield not only fundamental insights but also findings with high translational potential. Successfully translating these findings could lead to novel drugs targeting cysteine cathepsins for a wide spectrum of human diseases, including cancer, neurodegenerative, cardiovascular, and autoimmune disorders, as well as other inflammatory conditions.

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Ethics approval and consent to participate
This work does not contain any studies involving human and animal subjects.

Conflict of interest
The author of this work declares that he has no conflicts of interest.

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