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Cysteine cathepsins: From diagnosis to targeted therapy of cancer

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ABSTRACT

Cysteine cathepsins are a fascinating group of proteolytic enzymes that play diverse and crucial roles in numerous biological processes, both in health and disease. Understanding these proteases is essential for uncovering novel insights into the underlying mechanisms of a wide range of disorders, such as cancer. Cysteine cathepsins influence cancer biology by participating in processes such as extracellular matrix degradation, angiogenesis, immune evasion, and apoptosis. In this comprehensive review, we explore foundational research that illuminates the diverse and intricate roles of cysteine cathepsins as diagnostic markers and therapeutic targets for cancer. This review aims to provide valuable insights into the clinical relevance of cysteine cathepsins and explore their capacity to advance personalised and targeted medical interventions in oncology.

Contents

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1. Introduction

In cellular biology, cysteine cathepsins are enigmatic gatekeepers that orchestrate a symphony of proteolytic events crucial for cellular homeostasis, immune responses, and myriad physiological processes. The narrative of cysteine cathepsins dates back to the first half of the 20th century [1], with pioneering efforts paving the way for understanding these proteolytic enzymes. The groundbreaking discovery of papain, an enzyme extracted from papayas, marked the advent of cysteine protease research. This seminal finding, coupled with subsequent lysosomal enzyme-related investigations [2,3], laid the groundwork for the recognition of cysteine cathepsins as integral components of cellular degradation and turnover [4–6]. The realisation that they play pivotal roles in the immune response, apoptosis, and tissue remodelling unfolded gradually, whereas their involvement in health and disease became increasingly apparent, sparking a surge of research interest [6].

1.1. Cysteine cathepsins overview

According to the MEROPS database, bioinformatic analysis [7] confirmed the existence of 11 human cysteine cathepsins at the sequence level: cathepsins B, C (or J), F, H, K (or O2), L, O, S, V (or L2), W, and X (Z or P) [8]. They constitute the largest family of cathepsins within the C1a family of the CA clan and are characterised by a distinctive papain-like fold. They comprise the left and right domains harbouring a cysteine-histidine catalytic dyad positioned in the middle. The active protease features a catalytic Cys-His thiolate-imidazolium ion pair (Cys25-His159, papain numbering) located on opposite sides of the active site cleft [9], resembling a molecular scissor that facilitates substrate recognition and cleavage. Cysteine cathepsins typically exist as monomeric entities within the mass range of 20–35 kDa, depending on post-translational modifications. An exception to this monomeric pattern is tetrameric cathepsin C, which has a molecular weight of approximately 200 kDa [6].

Cathepsins B, C, F, H, L, O, and X are ubiquitously expressed in a spectrum of human tissues and cells, indicating their widespread presence and involvement in fundamental physiological processes. In contrast, cathepsins K, S, V, and W exhibit a more defined localisation, aligning with their specific functions and implying a more specialised role within distinct cellular compartments or processes (Table 1) [6,10].

Most cysteine cathepsins exhibit endopeptidase activity by which they cleave peptide bonds within the interior of a polypeptide chain. Cathepsins B and H possess molecular features that enable them to act as endo- and exopeptidases, whereas cathepsins C and X function strictly as exopeptidases. More precisely, cathepsin C is an aminodipeptidase and cathepsin X is a carboxymonopeptidase [6].

Highly efficient proteolytic cysteine cathepsins generally possess broad substrate specificity for the efficient degradation of a wide range of proteins. This allows them to function in several cellular processes, such as protein turnover and degradation, antigen processing and presentation, and cellular signalling. As supported by several studies, multiple cathepsins share common cleavage sites on their protein substrates. However, there are cases reported where certain substrates (collagen, osteocalcin, cytokines, and chemokines) are selectively targeted by only a subset of cathepsins [6,11–13]. The observed parallels in substrate preferences across cathepsins contribute to the perception of moderate specificity within this enzyme family. However, it is essential to acknowledge the diversity within the cathepsin family, as individual members may demonstrate distinctive substrate preferences. The substrate binding site known as S2 is arguably the most accurately characterised among its counterparts and uniquely qualifies as a 'pocket.' In conjunction with the S1 and S1′ sites, the S2 constitutes the Substrate Recognition Site (SRS). In terms of substrate specificity, most cysteine cathepsins exhibit a strong preference for small hydrophobic (Ala, Leu, Val and Ile) and aromatic amino acid residues (Phe and Tyr) at the P2 position. However, there are some differences observed among different cathepsins concerning P2 position specificities. For instance, cathepsin K displays a unique tolerance for proline in the P2 position, which is pivotal for its collagenolytic activity. Our previous study revealed that cathepsin L exhibits a higher preference for aromatic residues (Tyr and Phe) at the P2 position, whereas cathepsin S exhibits a higher preference for lysine. Furthermore, cathepsin B, which features a glutamic acid residue at position 245 (Glu245), displays a high specificity for arginine at the P2 position [6,9,14–16].

1.2. Activation, regulation, and physiological role of cysteine cathepsins

To avoid premature activation and potential cellular damage, cysteine cathepsins are initially synthesised as inactive zymogens or preprocathepsins in the endoplasmic reticulum (ER). These zymogens comprise a signal peptide, propeptide, and catalytic domain. The *N*-terminal signal peptide of 20–25 amino acids guides zymogens to the ER lumen, where the glycosylation with mannose occurs after signal peptide removal. Following mannose residue phosphorylation, procathepsins are directed to the *trans*-Golgi network by mannose-6-phosphate (M6P) receptors and are subsequently transported to lysosomes [17]. Within lysosomes, at acidic pH, the ultimate activation of procathepsins occurs through proteolytic cleavage of their *N*-terminal propeptides, leading to the formation of mature and active cysteine cathepsins. This activation occurs autocatalytically as a combination of unimolecular and bimolecular processes [18,19].

Following the activation, the enzymatic activity of cysteine cathepsins is meticulously regulated through multiple mechanisms including pH, compartmentalization, and endogenous protein inhibitors.

Cysteine cathepsins, predominantly found within the endo-lysosomal system, display optimal activity in slightly acidic, reducing environments, with an optimal pH range of 4.5–6.5 [6]. Most cysteine cathepsins function optimally under these conditions and are less stable at neutral pH [20,21]. It was observed that some cysteine cathepsin members exhibit additional catalytic activity at physiological pH and redox conditions although these are not favorable for their optimal activity. These properties of cysteine cathepsins suggest, that they might have alternative subcellular

Table 1
Expression Profile and Cancer Association of Cysteine Cathepsins cathepsins and their association with cancer type.

	Expression profile	Cancer type association
Cathepsin B	Ubiquitously expressed	Bladder cancer [104] Breast cancer [75,100,105,106] Cervical cancer [107] Colorectal adenoma and carcinoma [108,109] Endometrial cancer [110] Gastric cancer [111] Glioma [112] Lung cancer [113] Meningioma [114] Ovarian cancer [115] Oral squamous cell carcinoma OSCC [116,117] Pancreatic adenocarcinoma [118] Pancreatic ductal adenocarcinoma [82] Paediatric acute myeloid leukaemia [119] Prostate carcinomas [120] Salivary adenoid cystic carcinoma (SACC) [121] Thyroid malignancies [122]
Cathepsin C (also J and dipeptidyl-peptidase; 1 - DPPI)	Ubiquitously expressed	Brain tumour [123] Colorectal cancer [124] Liver cancer [125] Pancreatic cancer [95]
Cathepsin F	Ubiquitously expressed	(Paediatric) brain tumour [126] Cervical cancer [127] Gastric cancer [128] Lymphoma/leukaemia as a suppressor gene [129] Non-small cell lung cancer (NSCLC) [130] Thyroid cancer [131]
Cathepsin H	Ubiquitously expressed	Bladder cell carcinoma [104] Human colorectal cancer [132] Human hepatoma [133] Lung cancer [134] Prostate cancer [135]
Cathepsin K (also O2)	Osteoclasts, Epithelial cells [14,136]	Glioblastoma [137] Lymph node metastasis (LNM) [67] Melanoma [138] Oral squamous cell carcinoma (OSCC) [67] Osteosarcoma [139] Prostate cancer [140] Renal cancer [141] Breast cancer [142] Colorectal cancer [143] Cervical cancer [144] Gastric cancer [145]
Cathepsin L	Ubiquitously expressed	Breast cancer [146] Colorectal adenocarcinoma [147] Pancreatic adenocarcinoma and Pancreatic neuroendocrine cancer [73] Bladder cancer [104] Glioblastoma [148] Gastric cancer [88] Ovarian cancer [84] Renal cancer, testicular tumours, prostate, lung, thyroid [149] Chronic myeloid leukaemia [150] Paediatric acute myeloid leukaemia [119] Melanoma [151] Endometrial cancer [152] Nasopharyngeal carcinoma [153] Oral squamous cell carcinoma (OSCC) [154]
Cathepsin O	Ubiquitously expressed	Unknown
Cathepsin S	Antigen-presenting cells (dendritic cells, macrophages and B cells) [155]	Breast cancer and brain-related metastasis [78] Colorectal carcinoma [90,156] Brain cancer/Glioblastoma [157] Pancreatic cancer [95] Lung cancer [158] Prostate carcinomas [159] Glioma [160] Uveal/ocular cancer [160] Liver cancer [161,162] Thyroid cancer [163] Cervical cancer [164] Lymphoma [120] Gastric cancer [165,166] Renal cancer [167] Oral cancer [168]

Table 1 (*continued*)

	Expression profile	Cancer type association
		Bladder cancer [169]
Cathepsin V (also L2 or U)	Thymus and testis [170],	Breast cancer [172]
	Cornea and epidermis/skin [171]	Lung cancer [173]
	Thyroid, Heart, Brain [54]	Colorectal cancer, thyroid cancer, ovary cancer, endometrium cancer, thymic cancer [54]
		Squamous cell carcinoma [122]
Cathepsin W (also lymphopain)	Cytotoxic T cell type 1	Unknown
	Natural killer cells [174].	Pancreatic ductal adenocarcinoma [175]
Cathepsin X (also P or Z)	Ubiquitously expressed	Breast cancer [106]
		Glioblastoma [137]
		Colorectal [176]
		Gastric [177]
		Liver [178]
		Pancreatic cancer [93,95,179]

locations, outside of the endo-lysosomal system [4,22]. To improve cysteine cathepsin activity under unfavourable conditions, researchers use various polysaccharides [23] or substrates [20].

Various polysaccharides specifically modulate the activity of cysteine cathepsins. For example, the collagenolytic activity of cathepsin K is augmented by chondroitin-4 sulphate, chondroitin-6 sulphate and keratan sulphate, whereas it is inhibited by dermatan sulphate, heparan sulphate, and heparin. Interestingly, the collagenolytic activity of cathepsins S and L is reduced by chondroitin-4 sulphate [24–26], although the latter's effect may not be significant *in vivo*. Moreover, chondroitin sulphate inhibits the elastolytic activity of cathepsins V and K, but not that of cathepsin S [27]. Furthermore, the autocatalytic activation of cathepsins B, L, and S may occur even at neutral pH and is facilitated by several glycosaminoglycans and negatively charged surfaces [28–31].

Cysteine cathepsin's retained proteolytic activity outside the optimal pH range is associated with re-localisation to other compartments, such as the nucleus [32], cytosol [33] and extracellular compartment [34,35], thereby contributing to the expanding repertoire of cellular regions where these proteases may exert their influence [36].

The most simplistic mechanism of their translocation into other cellular regions is by leaking outside of lysosomes or by lysosomal membrane permeabilization (LMP), induced by a variety of stimuli including oxidative stress and lysosomotropic agents. The reason for this localisation can be found in their escape from standard trafficking routes into membranous intracellular vesicles or their leakage from these organelles. Two other escape routes are mRNA transcript variants, generated through the utilization of alternative promoters and/or alternative splicing translation that utilizes downstream in-frame start codons and secondly a phenomenon known as leaky scanning [37]. In these neutral-pH-compartments, cysteine cathepsins preserve their activity by binding to charged molecules or surfaces, such as glycosaminoglycans and even DNA, as well as by acidification [38].

An additional tier of regulatory mechanisms governing cysteine cathepsin activity involves the influence of endogenous inhibitors, including cystatins, stefins, kininogens, thyropins, and serpins. Inhibitors can be categorised into two groups: emergency and regulatory. Emergency inhibitors such as cystatins are typically located in cellular compartments separate from the enzyme. They swiftly trap proteases to form stable complexes that prevent undesired activity. By contrast, regulatory inhibitors modulate protease activity under normal physiological conditions and are often found in close proximity to their targets [39,40]. Among the extensively studied inhibitors are cystatins [family I25], which are further classified into the stefin [I25A] cystatin [I25B] and kininogen [I25C] subfamilies [8]. These inhibitors are characterised as competitive, reversible, and tight-binding and display the ability to differentiate between endo- and exopeptidases [6,40].

The predominant localisation of cysteine cathepsins in endolysosomal compartments aligns well with their recognised physiological roles in intracellular proteolysis, which is crucial for maintaining cellular homeostasis and facilitating cellular differentiation [41,42]. Although cathepsins generally degrade all proteins non-specifically during autophagy following the merging of lysosomes and autophagosomes, they also have more specific regulatory roles. One such example is cathepsin B, which cleaves the MCOLN1/TRPML1 calcium channel in the lysosomes during autophagy. This cleavage leads to the suppression of transcription factor EB (TFEB), the main transcription factor for cysteine cathepsins, thereby reducing the expression of lysosomal and autophagy-related proteins and serving as a feedback regulatory loop [43–45].

In addition, cysteine cathepsins play pivotal roles in the immune response, antigen processing and presentation, the processing and activation of various proteins and hormones, lysosomal death pathway, apoptosis, autophagy, ageing and other processes [12,42,46]. Their roles in both the innate and adaptive immune responses are especially important. In the context of innate immunity, lysosomal cathepsins have been demonstrated to cleave the ectodomains of Toll-like receptors (TLRs), leading to the recruitment of the adaptor protein MyD88 and the subsequent activation of TLR signalling pathways [47]. In adaptive immunity, various cathepsins participate in the proteolytic processing of antigens into short peptides and the degradation of the invariant chain, thereby facilitating adaptive immune responses [38]. The key enzymes in invariant chain processing are cathepsins L and S, with the latter playing a major role in the majority of organs and cell types [48], whereas cathepsin L is likely replaced by cathepsin V in humans [49,50]. However, cathepsins play redundant roles in antigen-processing for presentation to major histocompatibility complex II (MHC II) molecules.

Another physiological process in which cathepsins play a crucial role is bone remodelling. Thus, cathepsin K was found to be critical for the degradation of the extracellular matrix within bone, cartilage, and vascular tissues [14,35]. Regulated by the receptor activator of nuclear factor κB ligand (RANKL)-RANK signalling, its expression is predominantly associated with the degradation of collagen and other matrix proteins during osteoclast-mediated bone resorption, thereby ensuring the continuous turnover and maintenance of skeletal tissues [14,51,52].

On the other hand, nuclear cathepsin L has been recognised for its role in regulating the cell cycle by cleaving the CDP/Cux transcription factor, thereby influencing the G1/S transition [32]. In addition, during mouse embryonic stem cell differentiation, murine cathepsin L demonstrates the ability to cleave the *N*-terminal tail of

H3 histone, impacting gene regulation [53]. Notably, in humans, this regulatory role is likely mediated by cathepsin V [12,54] which is further supported by the fact that human cathepsin V, but not L, binds to DNA [55].

Several cysteine cathepsins (B, H, L, and S) were found to be involved in the regulation of apoptosis by targeting and activating the pro-apoptotic protein Bid and/or degrading prosurvival Bcl-2 homologues, thereby triggering the pro-apoptotic activity of Bak (BCL2 antagonist/killer) and Bax (BCL2 associated X, apoptosis regulator) [33,56–58]. The activation of Bak and Bax contributes to mitochondrial outer membrane permeabilization (MOMP), facilitating the release of pro-apoptotic factors and initiating the apoptotic cascade. Additionally, an X-chromosome-linked inhibitor of apoptosis (XIAP) has been identified as a target of cathepsins, indicating its potential role in mitochondrial caspase-dependent apoptosis [33].

In addition, cathepsins B, L, and K have important functions in the maintenance of constant hormone levels in the blood, for example, that of thyroid hormones [59].

In conclusion, cysteine cathepsins, with cathepsin B as a notable example, make valuable contributions to the wound healing process. Their involvement in extracellular matrix remodelling, as observed in scratch-wounding *in vitro* experiments [60], underscores their significance in promoting keratinocyte migration during wound repair.

2. Cysteine cathepsins in pathologies

Improper functioning and/or regulation of cathepsin activity can disrupt the delicate functional equilibrium, contributing to several pathologies including cardiovascular diseases, neurodegenerative disorders, obesity, bone disorders, arthritis, cystic fibrosis, infectious diseases, and cancer [46]. The aberrant activity of cysteine cathepsins in pathologies can arise from either excessive activity, often a consequence of dysregulated inhibition or expression leading to improper proteolytic processing, or the absence of activity, typically resulting from genetic abnormalities such as pycnodysostosis. Their increased activity is often associated with altered localisation, and in most pathologies, they are found in the extracellular milieu. However, most of these have been extensively discussed elsewhere [12,34,61,62], and we have focused primarily on cancer.

2.1. Cysteine cathepsins and cancer

The historical association of cysteine cathepsins with extracellular proteolysis in cancer dates back to more than four decades and was initially demonstrated for cathepsin B [63]. However, the precise roles of individual cysteine cathepsins in the extracellular milieu remain incompletely understood, given their intricate landscape. This complexity arises from several contributing factors, including broad substrate specificity, the presence of endogenous inhibitors, tumour-suppressive potential compensatory mechanisms, diverse cellular compositions within tumours, and effects unrelated to proteolytic function [62,64,65]. Aberrant cysteine cathepsin activity is commonly observed in cancers of both epithelial and mesenchymal origin, including several brain, lung, breast, colorectal, gastrointestinal, and melanoma cancers. Unfortunately, the upregulated expression of cysteine cathepsins in numerous cancer types is correlated with poor patient prognosis [62,66–68] (Fig. 1).

How is cathepsin overexpression regulated? It appears that the major pathways involved are transcriptional activation of TFEB or signalling pathways mediated by signal transducer and activator of transcription (STAT) proteins, specifically STAT3 and STAT6 [69,70]. Additionally, the expression and secretion of cysteine cathepsins can increase in response to stimulation by cytokines and interleukins (IL). Pro-inflammatory cytokines, including tumour necrosis factor (TNF), IL-1α, IL-1β, and interferon-gamma (IFNγ), have been shown to elevate the secretion of active cathepsin S from human chondrocytes, human smooth muscle cells, and mouse macrophages [71,72]. Cathepsins are actively released into the tumour microenvironment by diverse cell types, including tumour stromal cells, endothelial cells, tumour-associated macrophages (TAM), myoepithelial cells, fibroblasts, and other infiltrating cellular components at the tumour site [62,64]. The secretion of cysteine cathepsins in tumours is often accompanied by acidification of the extracellular milieu, a phenomenon that is particularly pronounced in the tumour microenvironment. Tumour-associated cells secrete large amounts of cathepsins into a mildly acidic environment, which is conducive to enzyme activity.

Cysteine cathepsins play important roles in many processes linked to tumourigenesis, including extracellular matrix degradation, angiogenesis, autophagy, apoptosis regulation, cancer invasion, metastasis, and disruption of cell-cell contacts. All of these have been well documented based on *in vivo* animal studies, *in vitro* studies involving cellular models, and clinical data [35,62,64–66,73–76]. However, the molecular signalling pathways involved in these processes are not well understood. Perhaps the most explored is the role of cysteine cathepsins in extracellular matrix (ECM) degradation, a significant contributor to cancer progression. Cathepsins have been found to degrade numerous ECM proteins including collagen, elastin, nidogen, fibronectin, laminin, osteonectin, and tenascin [35]. The degradation of ECM barriers facilitates the invasion of cancer cells into the surrounding tissues, fostering cancer dissemination. Therefore, increased cysteine cathepsin expression and the extracellular presence of these enzymes are associated with more aggressive cancer phenotypes and increased inflammation [34,62,64].

An important role of cathepsins was also discovered in the disruption of cell-cell contacts. This was primarily linked to the cathepsin sheddase function, wherein cathepsins L and S have been found to shed several cell adhesion molecules (CAMs) and junction adhesion molecules (JAMs) from the cell surface, including ALCAM, MCAM, and JAM-B, further contributing to tumourigenesis [77,78]. In addition, cathepsins have been found to shed several transmembrane receptors, such as transferrin receptors, neuropilins, ephrin-type receptors, and plexin B2, as well as for various cytokines, chemokines, and growth factors, all of which contribute to cancer growth and metastasis [34,79]. For instance, cathepsins B and L have been shown to alter the cell response to insulin-like growth factor-1 (IGF-1), a polypeptide that mediates somatic growth and can be dysregulated in various pathologies [80,81]. Cathepsin B also plays regulatory roles in cancer cell proliferation by influencing ERK/MAPK signalling in pancreatic [82] and colorectal cancers [83]. Similarly, Cathepsin L plays a significant role in cancer cell cycle regulation through its involvement in ERK/MAPK signalling [84] and the activation of the CDP/Cux transcription factor [32]. Notably, the loss of cathepsin B expression or cathepsin L activity leads to diminished cancer cell proliferation.

Several cathepsins, including B, L, and S, significantly influence angiogenesis. Specifically, cathepsin B has been identified as a regulator of angiogenesis in gliomas and colon cancer, potentially through its modulation of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and the activity of matrix metalloproteinase-9 (MMP-9) [85,86]. Cathepsin L facilitates angiogenesis directly by activating the angiogenesis-associated function of endothelial cells [87], or indirectly by modulating the CDP/Cux/VEGF-D pathway [88]. However, cathepsins L and, S, were also found to generate endostatin, a potent anti-angiogenic factor

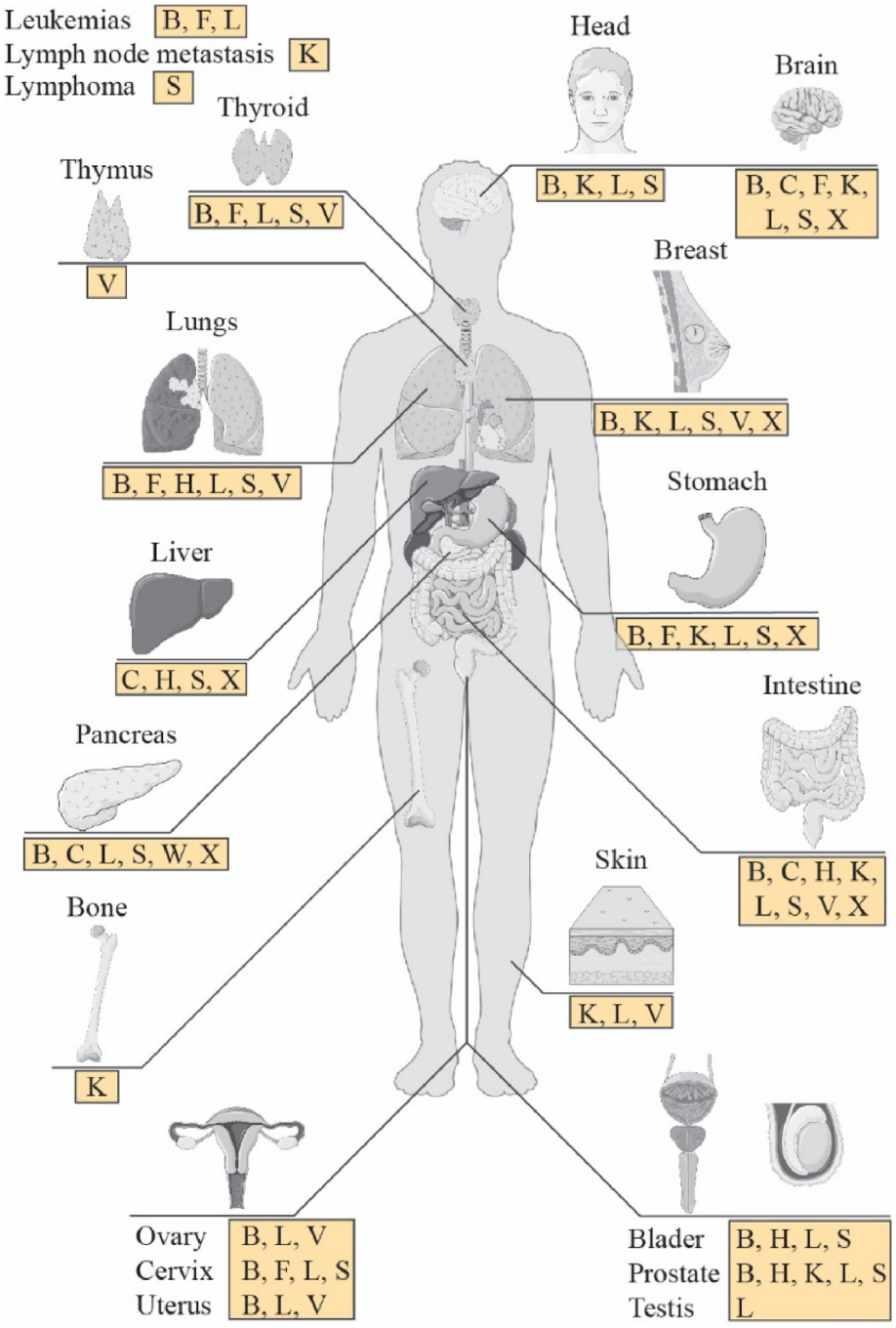

Fig. 1. Cysteine cathepsin association with tissue-specific cancers. Yellow squares indicate individual cathepsins linked to the associated type of cancer. References are within Table 1.

from collagen XVIII, possibly having dual roles [13,89]. Antibody-mediated inhibition of cathepsin S effectively impedes both angiogenesis and invasiveness in colorectal tumours [90].

In addition to their extracellular roles, cysteine cathepsins also play important intracellular roles in cancer. They serve as the primary effectors of protein catabolism and autophagy. This is crucial for the increased metabolic demands of proliferating cancer cells. Increased metabolic needs are supported by the activation of the mammalian target of rapamycin (mTOR) signalling pathway [91]. Another intracellular function is their potential involvement in the resistance to or triggering of apoptosis in cancer cells. Cathepsins were found to activate the BH3-only molecule Bid and degrade several anti-apoptotic proteins from the Bcl-2 family, thereby promoting apoptosis [33,56,58]. In addition, they have been suggested to be involved in other apoptotic signalling pathways [79]; however, it is not clear whether their pro-apoptotic functions could be used for therapeutic purposes without triggering significant side effects [92].

In many studies, cysteine cathepsins have exhibited contrasting roles depending on the tissue or cell type, as observed in several animal cancer models. In general, most cathepsins have potent tumour-promoting functions [62,73,75,93–97]. Conversely, in

other models, the same cathepsin may not contribute significantly to cancer progression, or may even have the opposite effect. The most pronounced effect was demonstrated for the loss of cathepsin L, exhibiting cancer promotion in *Ctsl*-deficient mice, which showed enhanced susceptibility to skin carcinogenesis compared to the wild-type, and in HPV16/Ctsl deficient mice, where it has been demonstrated that cathepsin L acts as an epidermal tumour suppressor [98,99]. Oppositely, in the pancreatic neuroendocrine tumour (PanNET) mouse model RIP1-Tag2, which was *Ctsl* deficient, it was shown that there is a decrease in cell proliferation in parallel with increased apoptosis [73]. In the same study of PanNET, they also confirmed a reduction in tumour burden, an increase in apoptosis, a reduction in proliferation and a decrease in angiogenesis when the mice model was deficient in *Catb* gene. Moreover, the loss of cathepsin B activity resulted in delayed progression and decreased proliferation of both the pancreatic intraepithelial neoplasia (PanIN) and the pancreatic ductal adenocarcinoma (PDA) which significantly improved survival in mice [82]. Similarly, the knock-out of the cathepsin B gene in mouse mammary cancer model MMTV-PyMT resulted in reduced cell proliferation in mammary carcinomas and their lung metastases [100]. Moreover, the loss of cathepsin B activity also contributes to cancer progression in the breast cancer model due to the compensatory increase in cathepsin X activity [101]. This highlights the critical need to consider specific cellular and tissue environments when investigating the role of cysteine cathepsins in tumour development. However, there have been numerous reviews on the role of cathepsins in cancer, and we do not discuss them in detail [62,64,95,96,102,103]. For a comprehensive overview of the association between individual cathepsins and various cancer types, please refer to Table 1 and the references within.

2.2. Cathepsins as diagnostic markers in cancer

Cathepsin-specific measurements in cancer diagnostics enhance the accuracy of identifying high-risk individuals and monitoring tumour progression, which are instrumental in the creation of personalised treatment plans. Numerous studies have demonstrated the changes in the levels of various cathepsins in biological samples [180], highlighting their immense clinical potential as biomarkers for early cancer diagnosis, which could enhance survival rates and improve prognosis. Recent findings using enzyme-linked immunosorbent assay (ELISA) have shown that in patients with oral squamous cell carcinoma (OSCC), the levels of salivary cathepsin B are significantly elevated compared to those in a control group. This increase was closely correlated with both histological grades and tumour sizes in patients with OSCC, suggesting that cathepsin B is an effective salivary biomarker for early cancer detection [117]. Serum cathepsin B concentrations were significantly elevated in patients with nasopharyngeal carcinoma (NPC) compared to healthy controls [181]. Additionally, it has been reported that serum cathepsin B levels are diagnostic markers of oesophageal cancer, showing higher concentrations in patient sera than in controls [182]. Elevated levels of cathepsin F in the serum and tissues were found to be significantly upregulated in patients with non-small cell lung cancer brain metastasis (NSCLC BM), making it a promising biomarker for early diagnosis. Changes in serum cathepsin F levels effectively reflected the therapeutic response in these patients, indicating its potential as a valuable tool for monitoring treatment efficacy [183]. Notably, cathepsin K is recognised as an immunohistochemical marker for categorising primary renal neoplasms, following the guidelines set by the International Society of Urological Pathology [184,185]. Under normal conditions, cathepsin K is prominently expressed in osteoclasts within the bone and exhibits low expression levels in other organs [186]. However, its expression is elevated in various cancer tissues. This overexpression is associated with metastatic cancer, underscoring its potential as a diagnostic and prognostic marker [187]. Recent immunoblotting experiments have revealed significant upregulation of urinary fucosylated glycoproteins, particularly cathepsin C and transferrin, in patients with small-cell lung cancer (SCLC). Moreover, the synthesis of cathepsin C in glioma cells was significantly greater than that in non-cancerous cells, with higher protein expression observed in high-grade gliomas, indicating that cathepsin C may serve as an efficient molecular target for diagnosing patients with brain tumours [123]. ELISA revealed the potential of serum cathepsin S as a promising biomarker for gastric cancer; elevated levels demonstrate diagnostic value correlating with tumour characteristics and serve as a prognostic indicator of overall survival [188]. Collectively, these findings indicate the potential of cathepsins as biomarkers of multiple cancer types.

2.3. Cathepsins as prognostic markers in cancer

Monitoring the overexpression of cathepsins in patients with cancer is crucial for predicting disease progression because it is typically associated with poor prognosis. However, in rare cases, cathepsin overexpression may be associated with a better prognosis, indicating a nuanced role of cathepsins in different cancer types [189]. This complexity is exemplified by cathepsin B, which is associated with tumour progression in various cancers including colorectal, breast, lung, pancreatic, and gastric cancers [65]. Recently, cathepsin B has been identified as a negative prognostic biomarker and therapeutic target in gliomas, where it is associated with immune cell infiltration and immunosuppression [190,191]. Moreover, it has been revealed that cathepsin B is overexpressed in the invasive front of salivary adenoid cystic carcinoma (SACC) compared to the tumour centre, showing a correlation with poor patient prognosis. Overexpression of cathepsin B, particularly in leader cells, is crucial for collective cell invasion, influencing extracellular matrix remodelling, and potentially defining its role as a key regulator in SACC progression [121]. The potential of cathepsin B as a prognostic marker for cancer can be enhanced by combining it with other biomarkers of interest. The ratio of serum cystatin C to cathepsin B offers significant prognostic value for predicting survival in patients with oesophageal carcinoma, with notably higher levels than normal indicating a more severe condition [182]. Furthermore, cathepsin K overexpression has been linked to a poor prognosis and increased lymph node metastasis in gastric cancer [192]. Similarly, in sarcomas, such as myxofibrosarcoma and undifferentiated pleomorphic sarcoma, its overexpression is associated with a higher risk of local recurrence and disease-specific mortality [193]. In salivary gland carcinomas (SGCs), their significant overexpression correlates with high-grade tumours, metastasis, and recurrence, reinforcing the potential of cathepsin K as a valuable prognostic biomarker across these diverse cancer types [194]. Additionally, using ELISA, it was observed that patients with metastatic lung cancer had an overexpression of cathepsin V in their serum compared to the control group, indicating a worse prognosis, further suggesting that cathepsin V could serve as a prognostic biomarker for lung cancer [173,195]. Moreover, recent research utilising the Cancer Genome Atlas-Liver Hepatocellular Carcinoma database identified cathepsin V as a gene of prognostic significance in liver cancer, where high cathepsin V expression correlates with poor prognosis, underscoring its potential as a biomarker for predicting outcomes in this cancer [196]. Similarly, in breast cancer, especially within the ER-positive luminal A subtype, cathepsin V expression has been linked to poor prognosis, highlighting its significance as a prognostic marker for this specific cancer subtype [172,197]. In contrast, a recent study

analysed the expression of cathepsin S across multiple human cancer datasets from The Cancer Genome Atlas, demonstrating its potential as a prognostic biomarker in various cancers. The study showed significant differential expression of cathepsin S across various cancers, with overexpression significantly linked to improved overall survival outcomes in colorectal, renal, melanoma, bladder, lung, lymphoma, sarcoma, and ovarian cancers, but worse outcomes in glioma and uveal cancer [160]. Using machine learning to identify biomarkers, it has been found that cathepsin W is significantly downregulated in pancreatic ductal adenocarcinoma, correlating with poor prognosis [175]. Field experts strongly advocate the use of machine learning to identify biomarkers in various cancers, highlighting its potential to significantly improve diagnostic accuracy in oncology. Consequently, close monitoring of both increases and decreases in cathepsin expression and understanding how these variations affect cancer prognosis are critical for accurately predicting the course of the disease.

3. Cathepsin imaging probes

Although the diagnostic approaches discussed above, including ELISA immunodetection, are highly specific, the use of cathepsin imaging probes potentially has additional diagnostic value. These probes specifically target the active protease form, allowing for a more detailed evaluation of their functional state and activity in biological systems, which enhances their diagnostic capabilities compared to traditional methods.

3.1. Molecular probes for cathepsin detection

In the field of probes and diagnostics, two primary categories are used to profile protease activity under *in vitro* and *in vivo* conditions: substrate- and inhibitor-based probes (activity-based probes; ABPs) [198,199]. Substrate-based probes generally require enzyme cleavage or activation for signal generation. They are often named based on their signal generation mechanisms, such as fluorogenic or Förster resonance energy-transfer (FRET) probes [200]. In contrast, ABPs, which originate from activity-based protein profiling [201], are designed to form covalent bonds at the catalytic site of the enzyme upon activation. These ABPs interact directly with the enzyme, offering a more precise measurement of their activity in various biological contexts (Fig. 2) [200,202].

Luminescent substrate-based imaging probes are widely used to detect cancer-associated proteins and their binding partners. In general, such probes are constructed using a molecular framework that includes a quenching group to suppress luminescence. Upon interaction with a specific cathepsin, the removal of the quenching group leads to a detectable light signal [202]. In 1999, Weissleder et al. pioneered the development of substrate-based probes activated by various lysosomal cathepsins. One of the first polymer probes developed was (Cy5.5)11-PL-methoxypolyethyleneglycol92 (CPGC), a graft copolymer designed for cathepsin B detection consisting of poly-L-lysine, methoxypolyethylene glycol succinate, and Cy5.5 dye. It showed low fluorescence because of the autoquenching of fluorophores whereas processing by cathepsins restored the majority of the quenched fluorescence [203,204]. Subsequently, the pan-cathepsin probe *Z*-FR-HMRG was designed using hydroxymethyl rhodamine green (HMRG) as a reporter. It provides a high tumour-to-background fluorescence ratio, which makes it particularly useful for real-time *in vivo* imaging of cathepsins using fluorescence endoscopy, proving to be especially effective in visualising intraperitoneally disseminated tumours [205]. Additionally, a luciferin-based bioluminescence probe, Val-Cit-AL, was developed for monitoring cathepsin B activity, demonstrating a “turn-on” of bioluminescence intensity at 590 nm with an excellent limit of detection (27 mU/L) *in vitro*. Time-course bioluminescence imaging of mouse breast cancer models indicated that Val-Cit-AL was sufficiently stable for short-term *in vivo* imaging [206]. According to recent data, the Val-Cit linker previously associated with cathepsin B can also be cleaved by other cysteine cathepsins [207]. Furthermore, two novel fluorescent/photoacoustic (FL/PA) probes, HCy-Cit-Val and HCy-Gly-Leu-Phe-Gly, were developed for *in vivo* imaging of cathepsin B. These probes demonstrated high sensitivity and selectivity and successfully monitored cathepsin activity *in vivo*, with HCy-Cit-Val showing superior properties owing to its higher catalytic efficiency, paving the way for its potential clinical use in early cancer diagnosis [208].

Although advances have been made in fluorogenic substrate-based probes for cathepsin detection, their use is often limited by background signal issues owing to incomplete quenching. To overcome this problem, FRET-based probes that utilise Förster resonance energy transfer have been developed. These probes enhance the signal-to-noise ratio through their efficient energy-transfer mechanism, leading to more precise and accurate detection of cathepsins [209]. Additionally, these probes can be modified to enhance certain features, such as increasing their uptake by cells or improving their ability to target tumours. In addition, the cathepsin *S*-selective FRET probe was modified by attaching palmitoic acid to the reporter. The lipid-modified probe showed a prolonged and more intense fluorescent signal in tumours than a similar probe without lipidation. This indicates the potential of non-invasive *in vivo* tumour detection using such modified probes [210,211].

Furthermore, the field of clinical diagnostics has notable contributions from activity-based diagnostics. ABPs with a classic architecture are built to include a reporter tag, a linker (or spacer), and an electrophilic warhead. This chemical moiety forms an irreversible covalent bond with the active enzyme in a mechanism-based manner [202]. In 2005, Blum et al. were the first to develop an ABP for cysteine cathepsins by introducing the concept of quenched fluorescent activity-based probes. These probes are initially non-fluorescent, but become fluorescent upon interacting with their target enzymes because of the displacement of the quencher group. They incorporated fluorophores into a peptide coupled with an acyloxymethyl ketone (AOMK) warhead and quencher to design cathepsin B- and L-selective GB111 probes [212]. In addition, the probe selectivity can vary with the recognition sequence. New substrates with high specificity for cathepsin L were identified using a positional scanning substrate approach with a hybrid combinatorial substrate library (HyCoSuL) containing many unnatural amino acids [100]. This work resulted in the discovery of the His-DThr-Phe(F5)-Cys (Bzl) sequence, which was modified with Cy5 dye through a 6-aminohexanoic acid spacer at the *N*-terminus and an (AOMK) warhead. This probe, named MP-cL3, distinguishes the activity of cathepsin L from that of other cathepsins [213]. A selective ABP for cathepsin B, MP-cB-2, was developed using a similar strategy. This probe was designed with a Cy5 dye at the *N*-terminus and an AOMK warhead at the *C*-terminus connected to a Cha-Leu-Glu (Bzl)-Arg sequence. The probe was employed to selectively label cathepsin B in eighteen tested cancer cell lines, establishing that this probe is highly suitable for various biological setups [214]. An example of this is qABP BMV083, which is composed of a triazole-based inhibitor, Cy5 dye, and a 2,6-dimethyl benzoic AOMK warhead with a QSY21 quencher. This probe, which exhibited selectivity for cathepsin S, enabled the visualisation of cancer *in vivo* in several murine models [215]. Later, the same probe was altered by introducing 2,3,5,6-tetrafluoro-substituted phenoxymethyl ketones (PMK) as electrophilic warheads alongside a sulfonated QSY21 dark quencher to improve pan-cathepsin labelling. This BMV109 probe was successfully employed

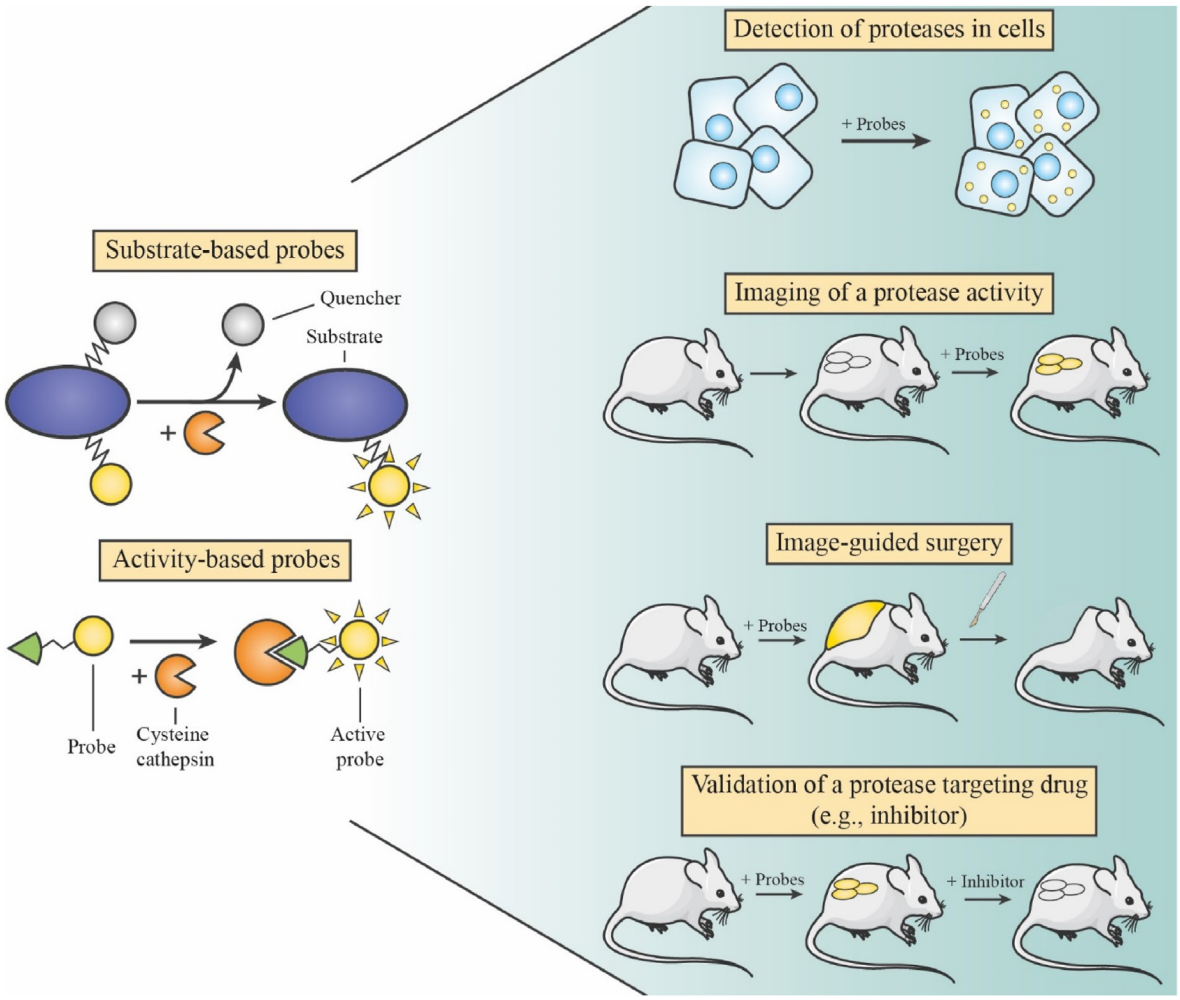

Fig. 2. Schematic representation of cysteine cathepsin selective molecular probes.

for *in vivo* imaging of breast cancer in mice, verifying the role of cathepsins in $CD68^{+}$ tumour-associated macrophages [216]. Notably, a switch in the reactive group on a particular scaffold can significantly alter the specificity of the probe, underscoring the importance of strategic design in achieving target selectivity.

In addition, Kramer et al. developed designed ankyrin repeat protein (DARPin) 8h6, a small antibody mimetic that is highly selective for cathepsin B and is characterised by its high affinity and fluorescent labelling. DARPin was effective in monitoring tumour-associated protein binding and was successfully used for minimally invasive *in vivo* imaging in two mouse models of breast cancer [217]. Recently, a nanoprobe based on cathepsin B and fibronectin overexpression was developed to provide a way to image and treat triple-negative breast cancer (TNBC) and treat it through photodynamic therapy. The probe binds to fibronectin-rich areas and is cleaved by cathepsin B, thereby releasing a photosensitive agent. This therapeutic agent allows for simultaneous fluorescence imaging, MR imaging, and photodynamic therapy at the same time [218]. Another activity-based photodynamic probe for the treatment and imaging of cancers was developed based on a carrier probe with a cathepsin-recognizing sequence that uses a QC-1 quencher to further reduce toxicity to nearby tissues. The quencher is released upon contact with overexpressed cathepsins in the tumour tissue, and the bacteriochlorin-based photosensitiser is activated [219]. Recently, a dual-modality theranostic agent, 68Ga/90Y-BMX2, targeting cathepsin B, was developed for cancer imaging and therapy, showing high affinity and specificity for binding to cathepsin B across various cancer cell lines. The probe demonstrated effective tumour imaging using PET and fluorescence and significant tumour growth inhibition in HeLa xenografts, highlighting its potential for clinical translation in cancer theranostics [220]. To utilise another imaging modality, functional CT imaging, gold nanoparticles were combined with cysteine cathepsin-targeted ABPs, resulting in high concentrations of contrast agents in the tumour microenvironment [221]. Similarly, iodinated probes targeting cysteine cathepsins have been developed. These probes included a targeting peptide specific to cathepsins, an electrophilic group for activity-dependent covalent binding, and dendrimer tags containing up to 48 iodine atoms. A variant with 48 iodine atoms was particularly effective for tumour imaging, demonstrating enhanced uptake and precise localisation in the tumour tissue [222].

3.2. Image-guided surgery

Solid tumours are still difficult to treat using non-invasive methods, requiring treatment by invasive surgical removal. The disadvantage of surgical removal is the uncertainty regarding the complete excision of tumour tissue while trying to preserve as much healthy tissue as possible. To resolve this problem, probes for

image-guided surgery have been developed [194]. Currently, a few probes used in mice have shown promising results, with one probe already being tested in humans. The activity-based VGT-309 probe is activated upon binding to tumour-specific- enzymes, including cysteine cathepsins. The first tests were performed on surgically removing breast cancer tumours in mice and improving the tumour removal accuracy (Fig. 2) [223]. Subsequently, the VGT-309 probe was tested for pulmonary tumour removal in humans and showed no toxicity at any of the studied doses and a high tumour-to-background signal ratio. Recent studies have shown promising results, for use in other tumour types [224]. There are also FDA-approved dyes that can be activated in the near-infrared (NIR) field. One of them is indocyanine green (ICG), which binds to a tumour-targeting- peptide named p28. ICG-p28 complex was used in *vitro* breast cancer model to evaluate its effectiveness. The probe was also tested in xenograft tumours expressing red florescent protein (RFP). The dye showed significant co-localisation with RFP, proving that it could specifically differentiate into tumour cells. The success of ICG-p28 has shown that operative tumour removal can be significantly improved [225]. In addition, the pan-cathepsin FRET probe, LUM015, was designed as a PEGylated cathepsin substrate that emitted fluorescence in the NIR range. It has shown promise for the intraoperative detection of microscopic residual cancer in solid tumours, effectively labelling tumours in both mouse models [226] and human clinical trials. Currently, the probe LUM015 is in the early stages of clinical trials (NCT04440982), for evaluation of the use of the LUM Imaging System in patients with breast cancer for intraoperative residual tumour detection. However, one of the main bottlenecks is the tissue penetration depth of NIR imaging techniques which currently measures approximately 10 mm. An increase in the viewing depth of NIR imaging techniques would greatly improve the efficiency and accuracy of surgical removal of cancerous tissues [223].

4. Development and clinical testing of cysteine cathepsin-based anticancer therapies

Advancements in drug development and medical research have led to a diverse array of cancer therapies, ranging from traditional treatments such as chemotherapy and surgical removal of cancerous tissue to more innovative approaches that utilise new markers overexpressed in the tumour microenvironment. Several innovative approaches for cancer treatment target cysteine cathepsins, as they are known to play a significant role in the development of cancerous tumours [62,64,103]. Their apparent importance has prompted the initiation of several preclinical and clinical trials exploring various therapies and diagnostic techniques based on cysteine cathepsins. Still, the transfer of results from basic research and preclinical testing to clinics has proven difficult. It is important to note that, to date, the majority of discovered compounds have only been tested in cell lines and animal models and have never been brought to clinical trials or the market for use in clinical therapies. This is partially due to inadequate efficacy or the occurrence of on- and off-target adverse effects [12,46]. In addition, preclinical data with cathepsin inhibitors [227–229] or gene ablation experiments in animal cancer models [62] have shown that blocking cathepsins in general only diminished or delayed tumour growth but did not clear the tumours, arguing against using inhibitors as monotherapies for cancer treatment. Currently, the most promising strategies for cancer treatment include mechanisms based on cysteine cathepsin inhibition using selective inhibitors, targeted drug delivery, and pro-drug activation that utilise cysteine cathepsin overexpression in the cancerous microenvironment [46].

4.1. Cysteine cathepsin inhibitors

The first endeavours to develop selective cysteine cathepsin inhibitors date back to the early 1980s and continue to be an important area of research for cancer treatment. Synthetic cysteine cathepsin inhibitors typically contain an electrophilic reactive group termed “warhead”, which enables a reaction with the nucleophilic thiolate part of the cysteine cathepsin active site and a peptide or nonpeptide moiety [230]. Depending on the type of reaction between the electrophilic warhead and cysteine cathepsin, we distinguish between reversible and irreversible mechanisms of inhibition [231]. The category of irreversible inhibitors includes compounds based on epoxysuccinates, epoxides and vinylsulfones, aziridines, azomethyl-, halomethyl- or acyloxymethyl ketones, azapeptides, hydroxamates, β-lactams, and α, β-unsaturated carbonyls. The most commonly used chemical groups as reversible inhibitors are aldehydes, methyl ketones, diketones, and nitriles [12,34,230].

One of the main warheads that provides useful information in the field of cathepsin is the epoxide. In 1978 Hanada et al. [232] isolated E−64 from *Aspergillus japonicus*. This epoxide is a non-selective inhibitor of all cysteine cathepsins, which greatly contributes to the research on these proteases. Based on the scaffold of E−64, the first selective inhibitors of cathepsin B, CA-030 and CA-074, were designed [233,234]. *In vivo* studies of human metastatic melanomas in a mouse lung cancer model showed that CA-074 significantly reduced tumour weight, delayed tumour growth, and significantly reduced lung metastasis [235]. In addition, Withana et al. [236] confirmed the cathepsin B inhibition potential of CA-074 in a mouse breast tumour model, where it significantly reduced bone metastasis, which was not observed with another epoxysuccinyl-based irreversible broad-spectrum cysteine cathepsin inhibitor, JPM-OEt. However, the use of irreversible inhibitors poses a concern owing to their potential side effects, especially in connection with the immune system. This has led to greater emphasis on the development of reversible inhibitors. Some of the reversible cathepsin B inhibitors that have been tested are nitroxoline and its derivatives. They bind to cathepsin B and reversibly inhibit its enzymatic activity [237]. Cathepsin B inhibition results in a notable reduction in ECM degradation, which in turn impairs tumour progression [238]. Another compelling inhibitor of cathepsin B is VBY-825. In the preclinical model of pancreatic islet cancer, it was found to significantly reduce the number of tumours and tumour growth, which was explained by the observed decrease in proliferation and increase in apoptosis of the treated tumour cells [228]. Despite the promising results obtained for VBY-825, research was discontinued when the developing company Virobay closed.

Selective inhibitors of cathepsin S also exhibit anticancer activity. In a preclinical *in vivo* study, Sevenich et al. [78] confirmed that the selective cathepsin S inhibitor VBY-999 reduced the tumour burden in the early progression of brain metastasis, but not in later stages. However, a similar fate was observed for VBY-999, which was produced by the same company as VBY-825. Promising inhibitory results were also obtained by a specific cathepsin S antibody Fsn0503 in combination with CPT-11 (irinotecan) when treating HCT116 colorectal carcinoma. Fsn0503 blocked cancer cell invasion, resulting in attenuated tumour growth [239]. 4-Morpholineurea-Leu-HomoPhe-vinylsulphone (LHVS) and *Z*-FL-COCHO (ZFL) have been used in preclinical trials of investigating the effect of cathepsin S inhibition on autophagy and apoptosis in glioblastoma. These findings indicate that the inhibition of cathepsin S by LHVS and ZFL induces autophagy, which is a prerequisite for the apoptosis of glioblastoma cells [240,241].

In addition to cathepsins B and S, the most pharmacologically

interesting cysteine cathepsin is cathepsin K, which is crucial for normal bone turnover, although this has not been extensively explored in cancer. However, in bone metastasis, cathepsin K is essential for the resorption of the protein matrix, and its inhibition could potentially result in an increase in bone mass and strength. This has garnered significant attention for the development of cathepsin K inhibitors with the potential to treat bone remodelling pathologies, including cancer [231,242]. A handful of cathepsin K inhibitors have entered clinical trials for osteoporosis and osteoarthritis; however, to date, only a few have addressed bone metastases [12,231]. The most extensively tested cathepsin K inhibitor is the covalent nitrile-based compound, odanacatib (MK-0822) [243]. Initially, odanacatib gained popularity as it rapidly advanced through phases 1 and 2 of clinical trials, showing promising results in reducing bone resorption markers in women with osteoporosis [242,244]. Further findings from the Long-term Odanacatib Fracture Trial (LOFT) and LOFT Extension study indicated that odanacatib may have severe cardiovascular side effects, including stroke, resulting in its discontinuation [242]. The research and development of odanacatib have highlighted the challenging nature of discovering new cysteine cathepsin-targeting therapies. Typically, the primary issues associated with all cysteine cathepsins, including cathepsin K inhibitors, involve unintended off-target effects on other cysteine cathepsins as well as on-target toxicity that influences many other physiological processes where cathepsin K is involved and is unrelated to bone resorption [12,46]. Simultaneously with the development of odanacatib, a few other cathepsin K inhibitors targeting bone metastases have been developed. These inhibitors are AFG-495 and L-235. A preclinical study conducted by, Le Gall et al. [245] showed that AFG-495 reduces breast cancer-induced osteolysis and skeletal tumour burden; similarly, Duong et al. [246] determined that L-235 has the capability to treat bone metastasis in breast cancer.

Another potential target for cancer treatment is cathepsin C, owing to its role in the activation of neutrophil serine proteases and inflammation. Although no direct studies have been conducted on cancer, the most advanced cathepsin C inhibitor, brensocatib, is currently in phase 3 clinical trials, and strategies for its use in cancer have been discussed elsewhere [247].

However, the use of cathepsin inhibitors in combined therapy with other established anticancer drugs such as demonstrated in combination with paclitaxel (Taxol) in a mammary gland cancer model substantially enhanced drug efficacy against primary and metastatic tumours [248], suggesting that cathepsin inhibition may be used in combination therapies.

5. Targeted delivery systems based on cathepsins

One way to minimise adverse effects and increase the efficiency of cysteine cathepsin-targeted therapies is to use nanoscale delivery systems that enable tissue-specific delivery and drug release. The most prominent systems include polymers, micelles, liposome carriers, protease-activatable prodrugs (PAPs), and antibody-drug conjugates (ADCs) (Fig. 3).

5.1. Polymer carriers

Polymer carriers are a method of drug delivery that uses polymers to increase the bioavailability of therapeutic substances or degradable polymers and linkers to attach therapeutic molecules. When the polymer encounters the correct enzyme, it is degraded and the therapeutic molecule is released into the surrounding environment. To inhibit cysteine cathepsin, cystatin was bound to a polymer carrier to increase its uptake by the tumour cells; this method resulted in increased intracellular cathepsin B inhibition [249,250].

N-(2-Hydroxypropyl)-methacrylamide (HPMA) polymers have been investigated as base molecules in therapeutics. The main concern was the accumulation of HPMA in different phagocyte system-associated tissues. The size of the polymer molecules directly affects the retention time and off-target effects. Smaller polymer blocks have a faster clearance time and still exhibit anti-tumour activity and longer retention times in tumour tissues. Polymers containing smaller blocks are more readily digested in the liver than in the spleen [251].

By attaching small molecules to polymers through cleavable linkers, toxic side effects are reduced and the specific release of small molecules can be achieved. HPMA is a polymeric base to which paclitaxel and gemcitabine can be attached. The efficacies of different polymer lengths and combinations of attached small molecules were tested [252]. Polyglutamate can be used as a biodegradable carrier polymer. Different therapeutic molecules that are activated or readily absorbed by cells, upon digestion of the polymer structure by cathepsins, can be attached to polyglutamate. Initial experiments used an NIR-photosensitive cytotoxic agent. It has also been proposed that multiple cytotoxic agents can be attached to achieve a multipronged therapeutic effect. Another strategy involves the attachment of large cytotoxic molecules that can enter cells only through endocytosis [253].

5.2. Micelle carriers

Another method for delivering cysteine-targeted drugs involves the use of micelle carriers. One of their most important advantages for drug delivery is their small size, which allows them to penetrate deeper into the tissues. Additionally, their hydrophobic cores allow for better delivery of certain therapeutics. In recent years, the focus has shifted to increasing their stability when administered, which is achieved by decreasing the critical micelle concentration [254]. Gao et al. [255] developed a self-assembling micelle carrier with an outer PEG shell and loaded with doxorubicin (DOX), a chemotherapeutic drug that inhibits cancer cell replication by inhibiting topoisomerase 2. Upon contact with cathepsin B, the outer PEG shell is cleaved, and hydrophobic therapeutics are released from the hydrophobic micelle core to attack the cancerous tissue. Another delivery method based on micelle carriers involves the direct crosslinking of small molecules into the micelle system. Using this method, improved drug loading (up to 58 %) and higher micelle purity can be achieved. Since the therapeutic is a part of the amphiphilic molecule, the micelle loading process and micelle assembly happen simultaneously. The main drawback of these small-molecule micellar systems is their short lifespan [256].

5.3. Liposome carriers

Compared to micelles, liposomes exhibit larger structures. Synthetic liposomes are composed of amphiphilic molecules organised into a lipid bilayer, mimicking the configuration of natural cellular structures. Liposomal carriers can encapsulate molecules within the interior space, integrate them into the lipid bilayer, or anchor them onto the surface of the bilayer [257]. The first cathepsin inhibitor-carrying liposomes were magnetoliposomes based on iron oxide-based nanoparticles, which enabled simultaneous monitoring by MRI and magnetic targeting, thereby serving as theranostic agents. This system was successfully used to improve the low bioavailability of JPM epoxide inhibitors in a mouse

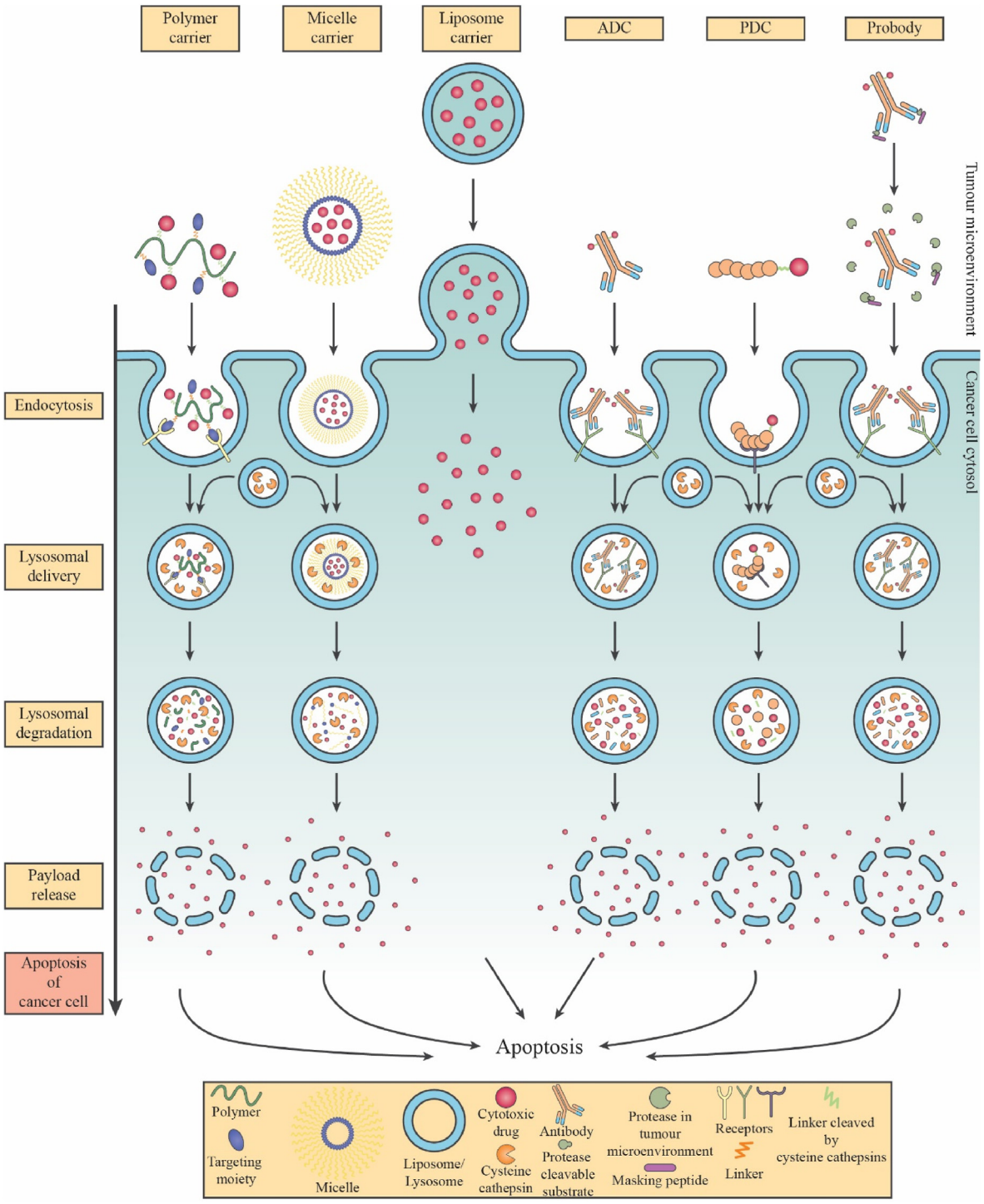

Fig. 3. Targeted nano-delivery systems based on cysteine cathepsins. Nanoscale delivery systems include polymer-carriers, micelle carriers, liposome carriers, antibody-drug conjugates (ADCs), peptide-drug conjugates (PDCs), and probodies that enable tissue-specific delivery and drug release. Nano-carriers are endocytosed by cancer cells and degraded in lysosomes, which release the cytotoxic payload, leading to apoptotic cells.

mammary gland cancer model [258]. Targeting was further improved when a lipidated epoxide-based selective cathepsin B inhibitor was inserted into the liposomal membrane to achieve active targeting. The liposomes also contained the MRI contrast agent. Thus, liposomal localisation can be monitored during drug delivery. Moreover, this system successfully delivered DOX to the targeted cells and was much more efficient than the classical, clinically approved doxyl system based on the liposomal delivery of DOX without targeting. In addition, this is the first study in which cathepsin was used as a target for targeted drug delivery and not as a therapeutic target [229]. Another example is stefin-A, an endogenous cathepsin inhibitor successfully used as a liposome-conjugated cathepsin-targeting ligand [259]. Another strategy is to create liposomes that are degraded by cathepsins to release therapeutic drugs into target cells. The GLFG peptide sequence can be used to create cathepsin-activating liposomes. The liposomes

can then be loaded with an anticancer therapeutic agent such as DOX [260].

5.4. Antibody-based therapies and drug conjugates

In addition to cysteine cathepsin inhibitors, other approaches utilise the activity of cysteine cathepsins and have gained significant attention for the clinical treatment of several tumours. Upregulation of cysteine cathepsins in several cancers has been used to develop protease-activating prodrugs (PAPs) [261]. PAPs are constructed by binding selected drugs to compatible carriers (mentioned above) using a specific linker that is recognised and cleaved by proteases, including cysteine cathepsins. As a result, PAPs facilitate targeted drug delivery to cancer sites which lowers negative side effects [262]. One of the fastest-growing classes of PAPs is antibody-drug conjugates (ADCs), wherein target-specific monoclonal antibodies (mAbs) are used as carriers of cytotoxic drugs [263]. Antibody-based immunotherapies are already an established field in cancer therapy with the promise of fewer side effects than traditional non-target-specific chemotherapy or radiotherapy. mAbs bind to specific antigens on the surface of cancerous cells, and the ADC is internalised via receptor-mediated endocytosis. Inside the cancer cell, the ADC is engulfed into a phagosome, where cysteine cathepsins cleave a specifically designed acid-labile linker and release the cytotoxic drug payload. This results in apoptosis of the cancerous cells. A recognised adverse outcome associated with ADCs is the bystander effect. Following apoptosis of a targeted cell, potent cancer-killing molecules are released into the neighbouring environment and are subsequently absorbed by nearby healthy cells. This leads to the unintended apoptosis of healthy cells [264,265]. To address the challenge of treating heterogeneous drug-resistant breast cancer, researchers developed a cleavable ADC with a dual payload. The findings from this study indicate that the efficacy of the dual-payload approach surpasses that of a combined antibody therapy in which the payloads are carried by separate antibodies [266]. Similar to drug-resistant breast cancer, pancreatic cancer is also difficult to treat. To determine a solution, Xu et al. [267] developed an ADC targeting TROP-2 positive pancreatic cancer cells carrying the potent antimitotic agent, monomethyl auristatin E (MMAE). This study showed that the nanobody-drug conjugate exhibited powerful antitumour activity, providing a novel way to battle TROP-2 positive pancreatic cancer cells [267].

Currently, there are 15 ADCs in the clinics (Table 2), of which 13 have successfully completed clinical trials and have been accepted by the Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) as efficient drugs to combat cancer. The remaining two are yet to be approved by the FDA or EMA, but are already available in the Japanese and Chinese markets. Eight of the 15 ADCs contain linkers that are recognised by proteases, three are pH-sensitive, two are non-cleavable, one is cleaved by glutathione, and the last has an undisclosed linker structure [264,268]. Valine–citrulline–p-aminobenzyl carbamate (ValCitPABC) is the most widely established linker used in clinical ADCs, that is cleavable by cysteine cathepsins. This peptide linker is recognised mainly by cysteine cathepsins B and L [269], which initiate the cleavage of the amide bond between citrulline and PABC, resulting in the release of the drug from the antibody. However, it was later shown that the ValCitPABC linker is also cleaved by cysteine cathepsins S and F [207]. Later, efforts were made to discover new linkers with better properties than those of ValCitPABC. Salomon et al. [265] designed and tested multiple newly designed linkers against the clinically established linkers. They discovered that the linkers Val-Gln (valine-glutamine), Leu-Gln (leucine-glutamine), and Phe-Gln (phenylalanine-glutamine) were cleaved at a faster rate by cysteine cathepsins than by ValCitPABC. At the same time, they also show high stability in plasma. However, this has not progressed beyond the scope of preclinical studies.

ADCs, antibody-drug conjugates; EMA, European Medicines Agency; mc-ValCitPABC, maleimidocaproyl-valine-citrulline-*p*-aminobenzyl carbamate; mc-GGFG-aminomethoxy, maleimidocaproyl-glycine-glycine-phenylalanine-glycine-aminomethoxy; Dxd, deruxtecan; SG3199, cytotoxic pyrrolobenzodiazepine dimer alkylating agent; CD, cluster of differentiation; HER2, human epidermal growth factor receptor 2; MMAE, monomethyl auristatin E; HL, Hodgkin lymphoma; sALCL, systemic Anaplastic Large Cell Lymphoma; DLBCL, diffuse large B-cell lymphoma; mUC, metastatic urothelial cancer; BC, breast cancer; NSCLC, non-small-cell lung cancer; GC, gastric cancer; GOJ, gastro-oesophageal junction cancer; B-cell prec. L, B-cell precursor leukaemia; CC, cervical cancer; UC, urothelial cancer; HCL, hairy cell leukaemia.

One of the main obstacles in the development of anticancer drugs is their ability to penetrate solid tumours. Because of their size, antibodies have a limited ability to penetrate solid tumours; thus, new approaches for treating solid malignancies are being developed. In addition to cysteine cathepsin-cleavable linkers in ADCs, they are also used in peptide-drug conjugates (PDCs). They represent an important class of therapeutic agents for effective prodrug-targeted delivery. PDCs are constructed by binding a cytotoxic drug to a carrier through a cleavable linker. However, PDCs utilise specific peptides instead of mAbs for the active targeting of a specific receptor on the surface of tumour cells. Peptides allow PDCs to penetrate the cancer cell membrane and release cytotoxic drugs inside the tumour cells [270,271]. The two main subclasses of peptides used in PDC research are cell-penetrating peptides (CPPs) and tumour-penetrating peptides (a class of tumour-homing peptides). CPPs can be non-selectively internalised by all cells, limiting their use in cancer research. In contrast, tumour-penetrating peptides are more interesting for cancer research because they can efficiently bind to specific tumour receptors and penetrate deep into cancerous tissues [272,273]. Small

Table 2
FDA and EMA approved ADCs with cysteine cathepsin-cleavable linkers.

ADC	Manufacturer	Linker	Payload	Target	Cancer
Brentuximab vedotin (Adcetris®)	Seagen, Takeda Pharmaceutical	mc-ValCitPABC	MMAE	CD30	HL; sALCL
Polatuzumab vedotin (Polivy®)	Genentech, Roche	mc-ValCitPABC	MMAE	CD79b	DLBCL
Enfortumab vedotin (Padcev®)	Astellas Pharma, Seagen	mc-ValCitPABC	MMAE	Nectin4	mUC
Trastuzumab deruxtecan (Enhertu®)	DaiichiSankyo, AstraZeneca	mc-GGFG-aminomethoxy	Deruxtecan, Dxd	HER2	HER2+ BC; NSCLC; GC/GOJ
Loncastuximab tesirine (Zynlonta®)	ADC Therapeutics	mc-ValCitPABC	SG3199, PDB dimer	CD19	large B-cell prec. L
Tisotumab vedotin (Tivdak®)[a]	Genmab, Seagen	mc-ValCitPABC	MMAE	Tissue factor	CC
Disitamab Vedotin (Aidixi®)[b]	RemeGen	mc-ValCitPABC	MMAE	HER2	UC; GC
Moxetumomab pasudotox (Lumoxiti®)	AstraZeneca	mc-ValCitPABC	PE38	CD22	HCL

[a] Approved only by FDA (Food and Drug Administration).
[b] Approved only by NMPA (National Medical Products Administration of China).

size of PDCs convey low immunogenicity but at the same time, they are limited by short half-life in blood [262,274].

Protease-activated antibody prodrugs, also termed probodies, have been developed to overcome the flaws in the treatment of solid tumours. They address the unwanted binding of antibody carriers to the target antigens in healthy tissues which causes undesirable side effects. Probody therapeutics contain a masking peptide bound to the *N*-terminus of the light chain of the antibody through a protease-cleavable linker that physically prevents the antibody binding. In the cancerous microenvironment, overexpressed cysteine cathepsins recognise and cleave specifically designed probody linkers, resulting in fully active antibodies [262,275,276]. The effectiveness of this method in clinical settings is evident in completed phase 1 clinical trials of probodies CX-2009 and CX-2029; however, the details of the mechanisms have not yet been disclosed [277,278].

6. Conclusions

In conclusion, cysteine cathepsins are major players in cancer progression. Therefore, they are considered as potential target molecules in cancer diagnosis and treatment. Although cathepsins are considered valuable diagnostic and prognostic markers for various cancers, they have never progressed beyond the preclinical evaluation stage. Approaches based on cathepsin targeting have recently attracted considerable attention. It seems that the classical inhibition of cathepsins is unlikely to succeed as a monotherapy; however, there is more hope for combination therapy, although it is not clear whether broad-spectrum inhibition or selective inhibition of an individual cathepsin would be more successful. Diagnostic imaging based on cathepsin-targeting has entered clinical trials and can be complemented with image-guided surgery. The most extensively exploited areas are antibody-drug conjugates and activatable prodrugs with several drugs, that use cysteine cathepsins activity, being approved for clinical use. Finally, the development of targeted drug delivery systems based on cathepsin-targeting is an emerging area of research. However, none of these studies has progressed beyond preclinical testing. Continued advancements in this field offer hope for new insights in cancer research.

Conflict of interest

The authors declare no conflict of interest

CRediT authorship contribution statement

Ana Ercegovič Rot: Writing – original draft, Conceptualization. **Matija Hrovatin:** Writing – original draft, Conceptualization. **Bor Bokalj:** Writing – original draft, Conceptualization. **Ernestina Lavrih:** Writing – original draft, Conceptualization. **Boris Turk:** Writing – review & editing, Funding acquisition, Conceptualization.

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