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Pharmacological Targeting of Lysosomal Cathepsin B and Its Impact
on Autophagy in Cancer Cells

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Hamza Al Sheikh BSc

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Abstract

Cancer cells depend on lysosomal degradation and autophagy to maintain metabolic homeostasis, remove damaged cellular components, and adapt to nutrient limitation and anticancer treatment. Cathepsin B (CTSB) is a lysosomal cysteine protease that contributes to terminal cargo degradation, but its biological effect varies according to enzyme localization, lysosomal integrity, pharmacological selectivity, and the functional role of autophagy in the investigated tumor model. This thesis aimed to critically evaluate how pharmacological targeting of lysosomal CTSB influences autophagic flux, lysosomal function, metabolic adaptation, and cancer-cell fate.

The work was conducted as a structured narrative review. PubMed/MEDLINE was used as the principal database, and the literature search was updated on 12 July 2026. Reference lists of relevant original studies and high-quality reviews were additionally screened. Priority was given to cancer studies assessing CTSB expression or activity together with autophagy-related outcomes, lysosomal function, and cellular or tumor responses. Evidence was organized into direct CTSB inhibition, indirect pharmacological modulation, complementary genetic manipulation, and CTSB-responsive drug-delivery strategies. Owing to substantial heterogeneity in cancer models, interventions, experimental assays, and endpoints, the findings were synthesized qualitatively.

The reviewed evidence indicates that preserved lysosomal CTSB activity can support productive autophagic flux, metabolic recycling, and tumor growth, most convincingly in pancreatic ductal adenocarcinoma. Conversely, reduced CTSB expression or activity frequently accompanied LC3-II and p62 accumulation, impaired cargo clearance, and increased treatment sensitivity in ovarian and other cancer models. However, most of these interventions also affected additional cathepsins, lysosomal acidification, membrane integrity, autophagosome–lysosome fusion, or intracellular drug distribution. They therefore demonstrate CTSB-associated lysosomal dysfunction more reliably than a selective CTSB-specific mechanism. Bortezomib-treated ovarian cancer cells provided comparatively strong evidence for a functional CTSB contribution because CTSB restoration partially improved cargo degradation and cell survival. The literature also revealed an opposite role following lysosomal membrane permeabilization: cytosolic CTSB may promote oncogenic-protein cleavage, mitochondrial apoptosis, ferroptotic damage, or other forms of lysosome-dependent cell death. Under these conditions, CTSB inhibition may antagonize rather than enhance anticancer therapy.

Overall, CTSB represents a conditional and compartment-dependent pharmacological target. Autophagosome or LC3-II accumulation must not be interpreted as autophagy induction without dynamic flux measurements, and total CTSB expression does not establish catalytic target engagement. Therapeutic development should therefore prioritize selective inhibitors capable of reaching the intended compartment, direct confirmation of CTSB inhibition, assessment of compensatory cathepsins, genetic validation and rescue, dynamic autophagy assays, and biomarker-guided selection of tumors in which CTSB-dependent lysosomal recycling is functionally important.

Zusammenfassung

Tumorzellen nutzen lysosomale Abbauprozesse und Autophagie, um ihre metabolische Homöostase aufrechtzuerhalten, geschädigte Zellbestandteile zu entfernen und sich an Nährstoffmangel sowie antineoplastische Therapien anzupassen. Cathepsin B (CTSB) ist eine lysosomale Cysteinprotease, die zum terminalen Abbau autophagischer Fracht beiträgt. Seine biologische Bedeutung hängt jedoch wesentlich von der subzellulären Lokalisation, der Integrität der Lysosomen, der Selektivität der eingesetzten Wirkstoffe und der funktionellen Rolle der Autophagie im jeweiligen Tumormodell ab. Ziel dieser Arbeit war es, kritisch zu bewerten, wie die pharmakologische Beeinflussung von lysosomalem CTSB den autophagischen Flux, die lysosomale Funktion, die metabolische Anpassung und das Schicksal von Krebszellen verändert.

Die Arbeit wurde als strukturierte narrative Literaturübersicht durchgeführt. PubMed/MEDLINE diente als zentrale Datenbank; die Literaturrecherche wurde am 12. Juli 2026 aktualisiert. Zusätzlich wurden die Literaturverzeichnisse relevanter Originalarbeiten und hochwertiger Übersichtsartikel durchsucht. Vorrangig berücksichtigt wurden onkologische Studien, in denen CTSB-Expression oder -Aktivität gemeinsam mit autophagiebezogenen Endpunkten, lysosomaler Funktion sowie zellulären oder tumorbiologischen Folgen untersucht wurden. Die Evidenz wurde in direkte CTSB-Hemmung, indirekte pharmakologische Modulation, ergänzende genetische Ansätze und durch CTSB aktivierbare Wirkstofffreisetzungssysteme gegliedert. Aufgrund der ausgeprägten Heterogenität der Tumormodelle, Interventionen, Untersuchungsmethoden und Endpunkte erfolgte eine qualitative Synthese.

Die ausgewerteten Daten zeigen, dass eine erhaltene lysosomale CTSB-Aktivität einen produktiven autophagischen Flux, metabolisches Recycling und Tumorstadium unterstützen kann; hierfür liefert das duktales Adenokarzinom des Pankreas die überzeugendste Evidenz. Umgekehrt gingen eine verminderte CTSB-Expression oder -Aktivität in Ovarialkarzinom- und weiteren Tumormodellen häufig mit einer Akkumulation von LC3-II und p62, gestörter Frachtdegradation und erhöhter Therapieempfindlichkeit einher. Die meisten Interventionen beeinflussten jedoch zugleich weitere Cathepsine, die lysosomale Azidifizierung, die Membranintegrität, die Fusion von Autophagosomen mit Lysosomen oder die intrazelluläre Wirkstoffverteilung. Sie belegen daher eine CTSB-assoziierte lysosomale Dysfunktion überzeugender als einen selektiven, ausschließlich durch CTSB vermittelten Mechanismus. Vergleichsweise starke funktionelle Hinweise ergaben sich aus Bortezomib-behandelten Ovarialkarzinomzellen, da die Wiederherstellung von CTSB die Frachtdegradation und das Zellüberleben teilweise verbesserte. Nach einer lysosomalen Membranpermeabilisierung kann CTSB jedoch eine gegensätzliche Funktion übernehmen: Im Zytosol kann es zum Abbau onkogener Proteine, zur mitochondrialen Apoptose, zu ferroptotischen Schäden oder zu anderen Formen lysosomenabhängigen Zelltods beitragen. Eine CTSB-Hemmung kann unter diesen Bedingungen die antitumorale Wirkung abschwächen.

CTSB ist somit kein universelles, sondern ein bedingtes und kompartmentabhängiges pharmakologisches Ziel. Eine Zunahme von Autophagosomen oder LC3-II darf ohne dynamische Fluxmessungen nicht als Autophagieinduktion gewertet werden; ebenso stellt die Gesamtmenge an CTSB keinen Nachweis einer funktionellen Hemmung der katalytisch aktiven Zielstruktur dar. Künftige Strategien sollten deshalb selektive, für das vorgesehene Kompartiment zugängliche Inhibitoren, den direkten Nachweis der CTSB-Hemmung, die Analyse kompensatorischer Cathepsine, genetische Validierung einschließlich Rescue-Experimenten, dynamische Autophagieassays sowie eine biomarkerbasierte Auswahl jener Tumoren kombinieren, in denen CTSB-abhängiges lysosomales Recycling tatsächlich funktionell relevant ist.

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1. Introduction

1.1 Background

Malignant cells develop within heterogeneous microenvironments characterized by intermittent hypoxia, restricted nutrient availability, oxidative stress, accumulation of damaged macromolecules and organelles, and exposure to anticancer therapies. These conditions create a sustained requirement for adaptive pathways that preserve metabolic homeostasis and intracellular quality control. Macroautophagy, hereafter referred to as autophagy, is one such pathway. It sequesters cytoplasmic material within autophagosomes and delivers it to lysosomes, where degradation generates amino acids and other metabolites that can be reused for energy production and biosynthesis [1,2].

In established tumors, autophagy can provide a survival advantage by maintaining mitochondrial function and supporting metabolic adaptation. Guo et al. demonstrated that oncogenic RAS increases basal autophagy and that disruption of autophagy leads to the accumulation of abnormal mitochondria, reduced oxidative metabolism, and impaired tumor-cell survival. Similarly, pancreatic cancer cells were shown to display elevated basal autophagy and dependence on this pathway for continued tumor growth. In genetically engineered lung-cancer models, acute autophagy inhibition impaired metabolic homeostasis and preferentially suppressed the growth of established KRAS-driven tumors [3–5].

Nevertheless, autophagy does not exert a uniformly tumor-promoting effect. Degenhardt et al. showed that autophagy supports tumor-cell survival during metabolic stress while simultaneously limiting necrosis and inflammation within tumors. Furthermore, experimental evidence from pancreatic tumor models demonstrated that the consequences of autophagy loss depend partly on the genetic background of the tumor, particularly p53 status. Autophagy should therefore be regarded as a context-dependent process whose biological effect varies according to tumor type, stage of development, oncogenic alterations, and environmental stress [6,7].

The completion of autophagic flux requires functional lysosomal fusion and efficient degradation of the delivered cargo. Lysosomal hydrolases are therefore essential for the terminal stage of this pathway. Cathepsin B is a lysosomal cysteine protease involved in intracellular protein degradation, but its biological effects may change when its expression, enzymatic activity, or subcellular localization is altered. Pharmacological targeting of cathepsin B may consequently influence lysosomal degradative capacity, autophagic flux, and cancer-cell survival. However, these outcomes cannot be predicted from cathepsin B inhibition alone because they may also depend on inhibitor selectivity, lysosomal accessibility, compensation by other cathepsins, and the functional role of autophagy in the investigated cancer model [1,8].

1.2 Scientific Rationale and Knowledge Gap

Although the lysosomal localization of cathepsin B provides a plausible mechanistic link to autophagic degradation, the available cancer evidence does not support one uniform outcome following alteration of cathepsin B activity. In ovarian cancer cells, bortezomib suppressed cathepsin B activity and disrupted autophagic flux, thereby increasing the cytotoxic effect of cisplatin [9]. Similarly, in multidrug-resistant HepG2/ADM liver cancer cells, the cycloartane triterpenoid ADCX reduced cathepsin B expression, impaired autophagic degradation, and

promoted apoptotic cell death [10]. These findings indicate that reduced cathepsin B function can contribute to late-stage autophagy impairment in some cancer models.

In contrast, experimental evidence from pancreatic ductal adenocarcinoma suggests that preserved cathepsin B proteolytic activity can support autophagic flux and tumor-cell metabolism. Jiang et al. demonstrated that cystatin B maintained cathepsin B activity, increased autophagic flux, and supported glycolytic metabolism and tumor progression [11]. This observation suggests that functional cathepsin B may contribute to the lysosomal recycling capacity required by metabolically active cancer cells. Therefore, pharmacological reduction of cathepsin B activity may weaken a survival pathway in selected tumors, but the relevance of this mechanism is likely to depend on the metabolic and lysosomal characteristics of the investigated cancer model.

Cathepsin B may also occupy a different position within autophagy-associated cell-death pathways. In SPARC-overexpressing primitive neuroectodermal tumor cells, inhibition of cathepsin B with CA-074Me reduced caspase-3 activation, PARP cleavage, and apoptosis without preventing the increase in LC3-II [12]. In this model, cathepsin B was not required for the initiation of the autophagic response but participated downstream in the transition from autophagy to apoptosis. Consequently, inhibition of cathepsin B could either impair lysosomal degradation or interfere with a death-promoting pathway, depending on the experimental context.

Interpretation is further complicated by methodological differences among studies. Some compounds directly inhibit cathepsin B, whereas others alter its expression, maturation, activity, or lysosomal environment indirectly. In addition, CA-074Me, which is frequently used as an intracellular cathepsin B inhibitor, may not retain complete selectivity for cathepsin B in living cells [13]. Studies that rely mainly on LC3-II accumulation or p62 changes without dynamic flux measurements also cannot reliably distinguish increased autophagosome formation from impaired autolysosomal degradation [1]. The central knowledge gap is therefore not simply whether cathepsin B affects autophagy, but how the targeting method, enzyme localization, inhibitor selectivity, autophagic flux, and final cell fate collectively determine the biological outcome. Addressing this gap constitutes the main scientific rationale of the present thesis.

1.3 Aim and Research Questions

The primary aim of this thesis is to critically evaluate how pharmacological targeting of lysosomal cathepsin B influences autophagy in cancer cells. Particular emphasis is placed on distinguishing productive autophagic flux from autophagosome accumulation caused by impaired lysosomal degradation and on determining how these different outcomes affect cancer-cell survival and death.

To achieve this aim, the thesis pursues the following objectives:

- to describe the molecular characteristics, maturation, regulation, and localization-dependent functions of cathepsin B;
- to explain the role of lysosomal cathepsin B within the autophagy–lysosome pathway;
- to evaluate direct cathepsin B inhibitors and pharmacological interventions that indirectly alter cathepsin B expression, activity, maturation, or lysosomal localization;

- to compare original experimental studies according to cancer model, targeting strategy, cathepsin B activity, autophagy assays, lysosomal effects, and final cell fate;
- to distinguish evidence of productive autophagy from late-stage autophagic blockade;
- to identify methodological limitations, conflicting findings, and factors that determine context-dependent outcomes;
- to assess the therapeutic opportunities and risks associated with targeting lysosomal cathepsin B in cancer.

The thesis addresses the following central research questions:

1. What role does lysosomal cathepsin B play in autophagic degradation in cancer cells?
2. How do direct and indirect pharmacological interventions affecting cathepsin B alter lysosomal function and autophagic flux?
3. Under which experimental and biological conditions does cathepsin B targeting cause impaired autophagic degradation, adaptive survival, or cancer-cell death?
4. How reliably do the available studies distinguish productive autophagy from late-stage autophagic blockade?
5. What pharmacological and methodological limitations currently restrict the therapeutic translation of cathepsin B-targeted strategies?

1.4 Methodology of Literature Search and Evidence Selection

This thesis was conducted as a structured narrative review of the scientific literature. PubMed/MEDLINE was used as the principal bibliographic database, and the search was updated on 12 July 2026. Reference lists of relevant original articles and high-quality reviews were additionally screened to identify studies that were not retrieved through the initial database search.

Search terms were combined using the Boolean operators AND and OR. The principal search concepts included “cathepsin B,” “CTSB,” “lysosome,” “autophagy,” “autophagic flux,” “cancer,” “tumor,” “CA-074,” “CA-074Me,” “cathepsin B inhibition,” “lysosomal dysfunction,” “lysosomal membrane permeabilization,” “apoptosis,” “prodrug,” and “nanocarrier.” Additional searches combined cathepsin B with individual cancer types, pharmacological compounds, autophagy markers, and experimental methods. Representative search combinations included:

- (“cathepsin B” OR CTSB) AND (autophagy OR “autophagic flux”) AND (cancer OR tumor);
- (“cathepsin B inhibition” OR CA-074 OR CA-074Me) AND autophagy;
- (“cathepsin B” OR CTSB) AND (“lysosomal dysfunction” OR “lysosomal membrane permeabilization”) AND cancer;
- (“cathepsin B” OR CTSB) AND (prodrug OR nanocarrier OR “drug delivery”) AND cancer.

English-language, peer-reviewed publications were considered. Priority was given to original experimental studies examining malignant cells, animal tumor models, or human tumor material. Studies were considered particularly relevant when they assessed cathepsin B activity or expression together with autophagy-related outcomes, lysosomal function, and cancer-cell survival or death. Reviews, systematic reviews, and methodological guidelines

were used to establish biological background, identify additional primary studies, and support the interpretation of autophagy assays and pharmacological limitations.

Studies were included when they addressed at least one of the following topics: the molecular and lysosomal biology of cathepsin B; the role of cathepsin B in cancer; direct pharmacological inhibition of cathepsin B; indirect pharmacological alteration of cathepsin B expression, maturation, localization, or activity; effects of cathepsin B modulation on autophagy or lysosomal degradation; or therapeutic systems that exploit cathepsin B activity for drug activation or delivery. Non-cancer studies were included only when they provided mechanistic evidence directly relevant to lysosomal cathepsin activity, autophagic degradation, inhibitor selectivity, or experimental interpretation. Their limited applicability to cancer was explicitly identified.

Publications were excluded when they did not distinguish cathepsin B from other proteases, lacked sufficient experimental information, addressed autophagy without a relevant connection to cathepsin B, or discussed cathepsin-responsive drug delivery without providing information relevant to the scope of the thesis. Conference abstracts, non-peer-reviewed material, and articles for which the relevant findings could not be adequately evaluated were not used as principal evidence.

The selected literature was organized into four evidence categories: direct pharmacological targeting of cathepsin B; indirect pharmacological modulation of cathepsin B; genetic manipulation of CTSL used as complementary mechanistic evidence; and cathepsin B-responsive drug-delivery strategies. Original studies concerning autophagy were compared according to cancer model, intervention, cathepsin B measurement, autophagy markers, use of dynamic flux assays, lysosomal effects, cell-fate outcomes, and methodological limitations. Particular weight was given to studies that combined cathepsin B activity measurements with multiple autophagy assays and functional experiments.

Because the available literature is heterogeneous in cancer type, treatment conditions, targeting methods, and experimental endpoints, the evidence was synthesized qualitatively rather than statistically. Conclusions were based on the consistency, directness, and methodological strength of the available evidence, with discrepancies and limitations discussed explicitly.

1.5 Structure of the Thesis

This thesis is organized into eight chapters that progress from the molecular foundations of cathepsin B and autophagy to the critical evaluation of pharmacological evidence and its therapeutic implications.

Following the introduction, Chapter 2 describes the molecular biology of lysosomal cathepsin B, including its structure, catalytic properties, biosynthesis, maturation, intracellular trafficking, endogenous regulation, and localization-dependent functions. Particular attention is given to the distinction between lysosomal, cytosolic, cell-surface-associated, and extracellular cathepsin B.

Chapter 3 presents the molecular organization of the autophagy–lysosome pathway in cancer. It explains the major stages of macroautophagy, autophagosome–lysosome fusion, lysosomal

degradation, autophagic flux, and the experimental methods used to distinguish productive autophagy from late-stage pathway blockade.

Chapter 4 examines the principal pharmacological strategies involving cathepsin B. These include direct enzyme inhibition, the experimental use and limitations of CA-074 and CA-074Me, lysosomal delivery and target engagement, as well as therapeutic approaches that exploit cathepsin B activity through activatable prodrugs and responsive drug-delivery systems.

Chapter 5 constitutes the central evidence-based chapter of the thesis. It compares original cancer studies investigating how direct or indirect alteration of cathepsin B affects autophagic flux, lysosomal function, metabolic adaptation, treatment response, and cancer-cell death.

Chapter 6 integrates the available findings into a mechanistic framework. It evaluates productive autophagic flux, impaired autolysosomal degradation, lysosomal membrane permeabilization, compensation by other lysosomal proteases, and interactions between cathepsin B, autophagy, and regulated cell-death pathways.

Chapter 7 critically discusses the strength and limitations of the current evidence, the selectivity of pharmacological tools, context-dependent therapeutic outcomes, combination-treatment strategies, and requirements for future clinical translation. Chapter 8 summarizes the principal conclusions and identifies the most important priorities for future research.

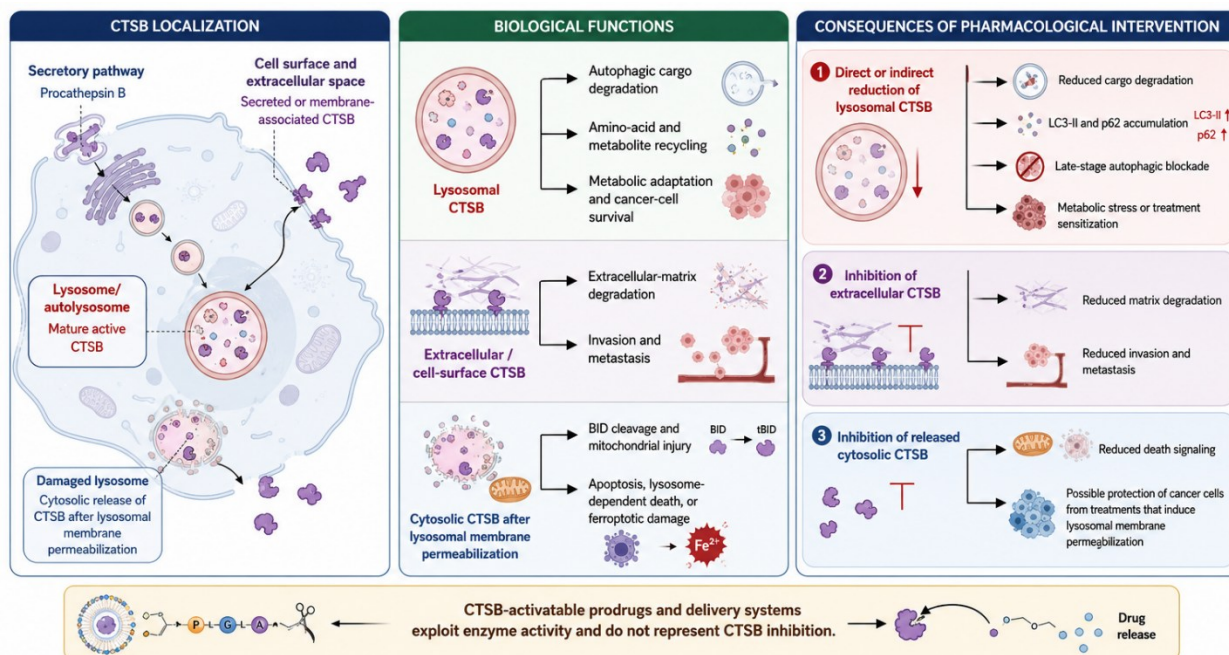


Figure 1. Compartment-dependent functions of cathepsin B and their relationship with autophagy and cancer-cell fate. Within intact lysosomes and autolysosomes, mature cathepsin B contributes to terminal cargo degradation and metabolic recycling. Cell-surface-associated and extracellular cathepsin B can promote extracellular-matrix degradation, invasion, and metastasis. Following lysosomal membrane permeabilization, released cathepsin B may gain access to cytosolic substrates and participate in apoptosis or other forms of lysosome-dependent cell death. Consequently, pharmacological inhibition may impair autophagic degradation, reduce invasion, or suppress treatment-induced cell death depending on the CTSB pool affected. Cathepsin B-activatable delivery systems exploit enzyme activity and are mechanistically distinct from CTSB inhibition. Conceptual synthesis based on references [9–13,30–32,94–108]. Created with BioRender.com.

Figure 1. Compartment-dependent functions of cathepsin B and their relationship with autophagy and cancer-cell fate

2. Molecular Biology of Lysosomal Cathepsin B

2.1 Structure and Catalytic Properties

Cathepsin B is a cysteine protease of the papain family. Its mature catalytic domain adopts the characteristic papain-like fold, consisting of two interacting domains separated by an active-site cleft. Catalysis depends principally on a cysteine and histidine located on opposite sides of this cleft. Structural analysis of human liver cathepsin B demonstrated that, although the enzyme shares the general architecture of other papain-like proteases, it possesses additional structural features that modify substrate access and catalytic behavior [14].

A defining feature of cathepsin B is the occluding loop, an approximately 20-residue insertion positioned over the primed side of the active-site cleft. This loop distinguishes cathepsin B from most related cysteine cathepsins and enables the enzyme to remove dipeptides from the C-terminus of protein substrates. Cathepsin B can therefore function both as an endopeptidase, cleaving peptide bonds within a substrate, and as a carboxydipeptidase, removing C-terminal dipeptides. Histidine residues within the occluding loop assist in recognizing the free C-terminal carboxyl group of suitable substrates [14,15].

Experimental modification of the occluding loop has confirmed its central role in determining catalytic specificity. Illy et al. showed that deletion of the central region of the loop eliminated exopeptidase activity while preserving endopeptidase function. The modified enzyme also displayed substantially increased affinity for cystatin C, indicating that the loop normally restricts access of protein inhibitors to the active-site cleft [15]. In a complementary mutagenesis study, disruption of the interactions that stabilize the loop produced a pronounced increase in endopeptidase activity. These findings demonstrate that the occluding loop is a flexible regulatory structure rather than a permanently fixed barrier [16].

Cathepsin B is initially produced as an inactive proenzyme. Crystallographic analysis of human procathepsin B demonstrated that its propeptide extends across the surface of the catalytic domains and blocks substrate access to the already formed active site. The occluding loop adopts a displaced conformation in the proenzyme, allowing the propeptide to occupy the catalytic cleft. This arrangement prevents inappropriate proteolytic activity before activation and illustrates that maturation involves both removal of the propeptide and structural rearrangement around the active site [17].

These structural properties are directly relevant to pharmacological targeting. Access to the catalytic cysteine, the conformation of the occluding loop, and the interactions formed within the substrate-binding cleft influence inhibitor binding and selectivity. Consequently, compounds described as cathepsin B inhibitors must be evaluated not only according to their ability to inhibit the purified enzyme but also according to whether they reach the relevant intracellular compartment and retain selectivity under cellular conditions. The pharmacological consequences of these structural characteristics are examined in detail in Chapter 4.

2.2 Biosynthesis, Maturation, and Lysosomal Trafficking

Cathepsin B is synthesized on the rough endoplasmic reticulum as an inactive preproenzyme. Its N-terminal signal peptide directs the newly synthesized polypeptide into the endoplasmic reticulum and is subsequently removed, generating procathepsin B. The remaining propeptide occupies the catalytic cleft and prevents premature proteolysis during transport through the secretory pathway. This inactive configuration is essential because unrestricted cathepsin B activity outside the appropriate degradative compartment could damage intracellular proteins and membranes.

During passage through the endoplasmic reticulum and Golgi apparatus, procathepsin B undergoes N-linked glycosylation and carbohydrate modification. A proportion of its oligosaccharide chains acquires mannose-6-phosphate residues that permit recognition by mannose-6-phosphate receptors in the trans-Golgi network. Receptor-bound procathepsin B is incorporated into transport carriers and delivered to endosomal compartments. Acidification within endosomes promotes dissociation of the enzyme–receptor complex, allowing the receptor to recycle while procathepsin B progresses toward late endosomes and lysosomes. Experimental alteration of the cation-independent Mannose-6-phosphate receptor-dependent trafficking contributes broadly to the intracellular delivery of lysosomal enzymes, while cancer-specific studies demonstrate that alteration of this system can modify the intracellular retention and distribution of cathepsin B [20,21].

Proteolytic maturation converts procathepsin B into an enzymatically active protease. Acidic conditions weaken the interaction between the propeptide and the catalytic domain, enabling removal of the inhibitory proregion. In vitro studies using recombinant human procathepsin B demonstrated that activation can occur through an intermolecular process in which initially active proenzyme molecules cleave additional procathepsin B molecules. Subsequent work confirmed that low intrinsic activity of the proenzyme can initiate this autocatalytic maturation process [18,19]. These findings explain how decreasing pH during endosomal maturation can promote conversion of an inactive precursor into an active lysosomal hydrolase.

Removal of the propeptide initially generates a catalytically active single-chain form of cathepsin B. Additional proteolytic processing within the endolysosomal system produces a double-chain form composed of heavy and light chains that remain associated. Both mature forms are enzymatically active, whereas the precursor form is substantially restricted by its propeptide. Consequently, measurements of total CTSB protein do not necessarily indicate the amount of catalytically active enzyme. Experimental studies should therefore distinguish CTSB expression from maturation and enzymatic activity, particularly when evaluating lysosomal function or autophagy.

Although mannose-6-phosphate-dependent transport represents an important route for cathepsin B delivery, its trafficking is not identical in every cell type. In HT-29 colon carcinoma cells, De Stefanis et al. detected substantial extracellular secretion of procathepsin B and concluded that mannose-6-phosphate receptor-dependent sorting played only a minor role in the intracellular segregation of lysosomal cathepsins in that model [22]. This observation demonstrates that malignant cells may use alternative or inefficient trafficking pathways and that findings from normal cells cannot automatically be transferred to all cancer models.

Altered trafficking can have direct biological consequences in cancer. Rose et al. showed that intracellular retention of cathepsin B through the cation-independent mannose-6-phosphate/insulin-like growth factor II receptor was required for the transformation of endothelial cells by Kaposi sarcoma-associated herpesvirus. Disruption of this trafficking relationship changed intracellular cathepsin B activity and impaired the transformed phenotype [21]. In other tumors, inefficient lysosomal targeting may instead increase secretion or redistribution of procathepsin B toward the cell surface and extracellular compartment. Thus, CTSB biosynthesis, maturation, and localization must be evaluated as interconnected processes rather than interpreting expression level alone.

This distinction is particularly important for the present thesis. A pharmacological compound may reduce CTSB catalytic activity without changing protein abundance, prevent proenzyme maturation, interfere with lysosomal acidification, or alter intracellular trafficking. Each mechanism can produce a different effect on lysosomal degradation and autophagic flux. Therefore, the precise step affected by a pharmacological intervention must be identified before an autophagy-related outcome can be attributed specifically to cathepsin B inhibition.

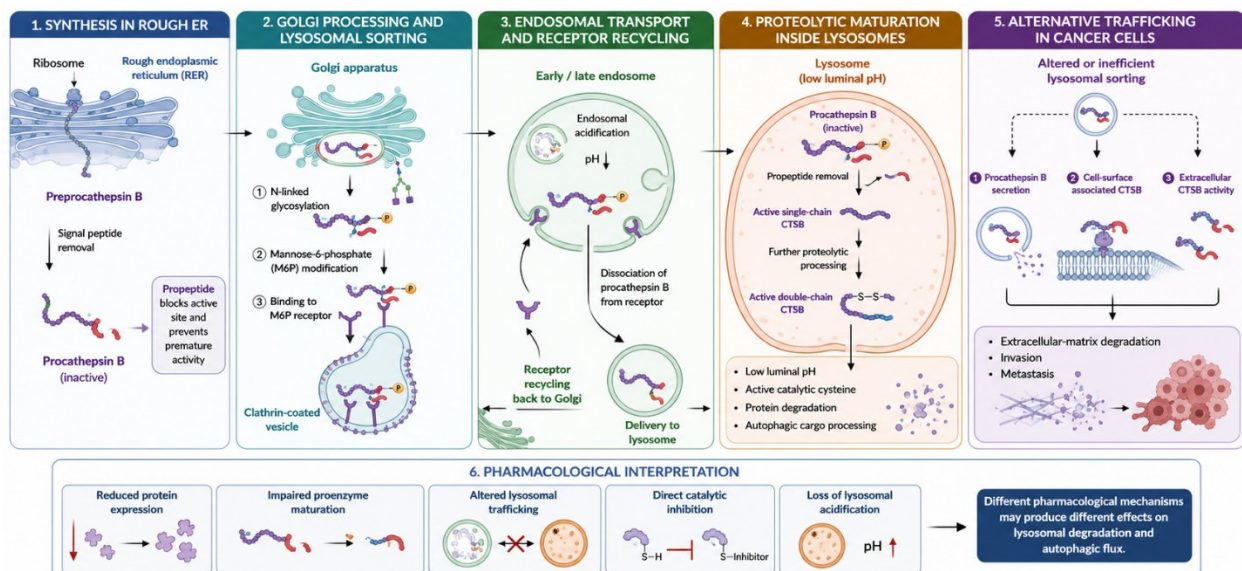


Figure 2. Biosynthesis, maturation, and intracellular trafficking of cathepsin B. Cathepsin B is synthesized in the rough endoplasmic reticulum as preprocathepsin B. Removal of the signal peptide generates inactive procathepsin B, whose propeptide prevents premature catalytic activity. Following N-linked glycosylation and mannose-6-phosphate-dependent sorting in the Golgi apparatus, procathepsin B is transported through endosomal compartments toward lysosomes. Endosomal acidification promotes receptor dissociation and facilitates proteolytic removal of the propeptide. The resulting active single-chain enzyme may undergo further processing to form the active double-chain form. In cancer cells, altered intracellular sorting may increase secretion or redistribution of cathepsin B toward the cell surface and extracellular environment. Changes in CTSB expression, maturation, trafficking, and catalytic activity therefore represent mechanistically distinct pharmacological effects. Conceptual synthesis based on references [18–22]. Created with BioRender.com.

Figure 2. Biosynthesis, maturation, and intracellular trafficking of cathepsin B

2.3 Regulation by pH, Redox State, and Endogenous Inhibitors

The proteolytic activity of cathepsin B is regulated by the physicochemical conditions of its surrounding compartment. Lysosomal acidification promotes proenzyme maturation and provides an environment favorable for substrate cleavage. Nevertheless, cathepsin B should not be considered completely inactive outside acidic lysosomes. Comprehensive substrate profiling demonstrated that the enzyme possesses both dipeptidyl carboxypeptidase and endopeptidase activities across acidic and near-neutral pH conditions. The relative catalytic efficiency and amino-acid preferences surrounding the cleavage site, however, changed according to pH [23]. These findings provide a biochemical basis for the ability of cathepsin B to process different substrates after redistribution from lysosomes to the cytosol, cell surface, or extracellular environment.

Redox conditions provide an additional level of regulation because cathepsin B depends on a catalytic cysteine residue. Experimental perturbation of the thioredoxin system demonstrated that oxidative stress can alter cathepsin activity without producing equivalent changes in protein abundance. In nutritionally stressed SH-SY5Y cells, thioredoxin reductase inhibition by auranofin caused lysosomal membrane permeabilization and increased cathepsin B activity despite the absence of a corresponding increase in cathepsin B protein. Cathepsin L showed a different response, with increased protein abundance but no comparable increase in catalytic activity [24]. Although this was not a cancer model, it demonstrates mechanistically that redox state, enzyme activity, and protein expression must be evaluated separately.

Cathepsin B activity is also controlled by endogenous inhibitors belonging to the cystatin family. Intracellular stefins, including stefin A and stefin B, and secreted type 2 cystatins, particularly cystatin C, restrict inappropriate cysteine-protease activity. Their interaction with cathepsin B is structurally more complex than simple occupation of an unobstructed catalytic cleft. Kinetic analysis demonstrated that cystatin C inhibition occurs through an initial association followed by a conformational step in which the inhibitor displaces the cathepsin B occluding loop [25]. Structural analysis of the cathepsin B–stefin A complex similarly showed that stefin A moves the loop sufficiently to gain access to the active-site cleft [26]. Thus, the occluding loop influences not only substrate specificity but also susceptibility to physiological inhibitors.

Although cystatin C is predominantly secreted, it can also regulate intracellular cysteine cathepsins after cellular uptake. In several human cancer cell lines, including breast, epidermoid, and pancreatic cancer cells, extracellular cystatin C was internalized through an active process and remained functionally capable of inhibiting cysteine proteases. Further experiments demonstrated vesicular localization of internalized cystatin C and reduced intracellular activity of its target enzymes [27,28]. These observations indicate that the balance between cathepsin B and cystatin C can be regulated across both extracellular and intracellular compartments.

The cathepsin–cystatin relationship may itself become dysregulated in cancer. In HCT116 colorectal cancer cells, cystatin SN interacted with cystatin C and weakened its inhibitory effect on cathepsin B. Restoration of cathepsin B activity was accompanied by increased extracellular proteolysis and cancer-cell invasiveness [29]. Conversely, the presence of a cystatin does not always predict reduced cathepsin B function. As discussed in Section 1.2, cystatin B was reported to sustain cathepsin B proteolytic activity and autophagic flux in pancreatic ductal adenocarcinoma [11]. These apparently opposing findings demonstrate that net cathepsin B activity is determined by the identity, localization, concentration, and molecular interactions of individual cystatins rather than by their expression alone.

The regulation of cathepsin B therefore occurs at several interconnected levels. Lysosomal pH influences maturation and substrate preference, redox conditions affect catalytic competence and lysosomal integrity, and endogenous inhibitors control access to the active site. For pharmacological studies, total CTSB protein abundance is consequently an insufficient indicator of functional inhibition. Reliable interpretation requires measurement of catalytic activity together with lysosomal localization, environmental conditions, and the balance between cathepsin B and its endogenous regulators.

2.4 Localization-Dependent Functions of Cathepsin B

The biological function of cathepsin B is strongly determined by its subcellular and extracellular localization. The principal functional pools include lysosomal cathepsin B, cytosolic cathepsin B released after lysosomal membrane permeabilization, cell-surface-associated cathepsin B, and secreted cathepsin B in the extracellular environment. Although these pools originate from the same protein, they gain access to different substrates and can therefore produce substantially different biological outcomes.

Within intact lysosomes and autolysosomes, cathepsin B contributes to controlled protein degradation as part of a broader network of lysosomal hydrolases. In this compartment, its function supports intracellular recycling and the degradation of material delivered through endocytic and autophagic pathways. Experimental evidence from pancreatic ductal adenocarcinoma demonstrated that preservation of cathepsin B proteolytic activity was associated with increased autophagic flux and glycolytic metabolism, illustrating how the lysosomal pool may support the metabolic requirements of cancer cells [11]. However, cathepsin B is not solely responsible for lysosomal proteolysis, and its contribution depends on the activity of other cathepsins and hydrolases operating within the same compartment.

In malignant cells, altered intracellular trafficking may redistribute cathepsin B toward the plasma membrane and pericellular space. Victor et al. detected enzymatically active cathepsin B in caveolae-enriched membrane fractions of SUM149 inflammatory breast cancer cells. Treatment with the poorly cell-permeable inhibitor CA-074 reduced pericellular degradation of type IV collagen and decreased tumor-cell invasion, supporting a functional role for cell-surface-associated cathepsin B rather than exclusively intracellular enzyme activity [30]. This study is particularly informative because it combined measurement of active cathepsin B with live-cell matrix-degradation and invasion assays.

Extracellular cathepsin B can contribute to tumor progression by degrading matrix proteins and cooperating with other proteolytic systems. Evidence from the immunocompetent 4T1.2 breast cancer model further demonstrated that RNA-interference-mediated CTSE reduction decreased collagen I degradation and bone metastasis. Systemic administration of CA-074 similarly reduced metastatic burden, whereas a broader cysteine-cathepsin inhibitor did not reproduce the same effect [31]. These findings indicate that extracellular or pericellular cathepsin B can contribute causally to metastatic progression in selected tumor models, although they do not establish that the same mechanism operates in every cancer type.

A fundamentally different outcome may occur when lysosomal membrane integrity is disrupted. Lysosomal membrane permeabilization allows mature cathepsin B to enter the cytosol, where it encounters proteins that are normally protected from lysosomal proteolysis. In chronic myelogenous leukemia cells, imatinib-induced lysosomal membrane permeabilization caused cytosolic redistribution of cathepsin B and proteolytic degradation of the BCR–ABL oncoprotein. Pharmacological or genetic interference with cathepsin B reduced this effect, indicating that released cathepsin B contributed directly to the elimination of the leukemia cells [32].

Cytosolic cathepsin activity has also been implicated in the killing of treatment-resistant glioma cells. Di Piazza et al. showed that infection with oncolytic parvovirus H-1 induced lysosomal alterations and cytosolic activation of cathepsins in cisplatin- and TRAIL-resistant glioma cells. Interference with cathepsin activity reduced cell killing, demonstrating that

lysosomal proteases participated functionally in the death response [33]. Similarly, in SPARC-overexpressing primitive neuroectodermal tumor cells, cathepsin B inhibition reduced apoptosis without preventing the upstream autophagic response [12]. Together, these studies show that cytosolic cathepsin B can participate in treatment-induced cell death rather than functioning exclusively as a tumor-promoting enzyme.

The extent and timing of lysosomal membrane permeabilization are important for interpreting these findings. Partial release may permit selective cleavage of cytosolic substrates, whereas extensive lysosomal damage can produce broader proteolytic injury. The outcome is also influenced by the cancer model, the death stimulus, the presence of endogenous inhibitors, and the simultaneous release of other lysosomal enzymes. Therefore, detection of cathepsin B outside lysosomes does not by itself prove that the enzyme is the principal mediator of cell death; functional inhibition, genetic manipulation, and measurement of catalytic activity are required to establish causality.

Localization is also critical for pharmacological interpretation. A poorly permeable inhibitor may preferentially affect extracellular or cell-surface cathepsin B and reduce invasion without substantially inhibiting lysosomal proteolysis. Conversely, a cell-permeable compound may reach lysosomes, affect multiple cysteine cathepsins, impair autophagic degradation, or alter lysosomal membrane stability. The biological meaning of “cathepsin B inhibition” therefore remains incomplete unless the relevant enzyme pool, intracellular access, catalytic target engagement, and resulting cellular process are identified.

2.5 Cathepsin B Dysregulation in Cancer

Cathepsin B dysregulation in cancer should not be defined solely as an increase in total protein expression. Malignant transformation may alter CTSB transcription, protein abundance, proenzyme maturation, catalytic activity, intracellular trafficking, secretion, and subcellular localization. These changes are not biologically equivalent. Increased lysosomal cathepsin B may enhance intracellular degradation, whereas redistribution toward the cell surface or extracellular compartment may promote pericellular proteolysis. Consequently, the pathological significance of cathepsin B depends on the amount of catalytically active enzyme, its cellular source, and the compartment in which it operates.

Evidence from human tumors supports an association between increased cathepsin B and cancer progression, although the strength of this association differs among studies. In colorectal carcinomas, Campo et al. reported that cathepsin B expression correlated with tumor progression and shorter patient survival [34]. In breast cancer, Maguire et al. detected higher cathepsin B levels in primary and metastatic tumors than in benign fibroadenomas. High tumor levels were associated with shorter disease-free and overall survival, but cathepsin B was not an independent prognostic factor after adjustment for established clinical variables [35]. This distinction is important because an association with poor outcome does not by itself demonstrate that cathepsin B independently drives disease progression.

More direct causal evidence has been obtained from genetically modified tumor models. In the MMTV–PyMT mammary cancer model, Vasiljeva et al. showed that cathepsin B deficiency delayed tumor onset, reduced tumor growth, and decreased pulmonary metastatic burden. Experiments separating tumor-derived from host-derived enzyme activity indicated that cathepsin B produced by both malignant cells and macrophages could contribute to mammary cancer progression [36]. These findings demonstrate that the tumor-promoting

cathepsin B pool may originate from more than one cellular population within the tumor microenvironment.

However, the relative contribution of cancer-cell-derived and stromal cathepsin B is not identical in every experimental setting. Bengsch et al. selectively overexpressed human CTSB in cancer cells or stromal cells within a murine breast cancer model. Overexpression in cancer cells increased extracellular matrix degradation, collective invasion, and tumor growth, whereas overexpression in the host stroma or macrophages did not produce an equivalent additional effect [37]. The study also found that increased tumor volume was not explained by a simple increase in proliferation, indicating that CTSB promoted progression mainly by enhancing matrix proteolysis and invasion. Thus, the importance of the enzyme source depends on whether the experiment examines physiological expression, genetic deficiency, or forced overexpression.

Cathepsin B also contributes to tumor progression in cancer models outside the breast. In genetically engineered models of pancreatic ductal adenocarcinoma, constitutive CTSB loss delayed the progression of pancreatic intraepithelial neoplasia and invasive carcinoma and prolonged survival. Cathepsin B-deficient tumor cells displayed reduced proliferation and MAPK signaling, while restoration of CTSB reversed these effects. The study additionally identified changes in the active forms of cathepsin L, suggesting that the biological consequences of CTSB loss may involve interactions with other lysosomal proteases rather than the isolated absence of one enzyme [38].

These studies demonstrate that cathepsin B dysregulation can contribute to several cancer-associated processes, but they do not support a universal mechanism applicable to all tumors. Clinical expression studies primarily establish association, whereas genetic deletion, cell-specific overexpression, activity inhibition, and rescue experiments provide progressively stronger evidence of causality. Furthermore, effects on extracellular invasion cannot automatically be interpreted as evidence of altered autophagy. For the present thesis, studies of CTSB dysregulation therefore provide the cancer-biological foundation, while conclusions concerning autophagy require separate evidence showing changes in lysosomal degradation, dynamic autophagic flux, and cancer-cell fate.

2.6 Functional Redundancy with Other Lysosomal Cathepsins

Cathepsin B functions within an interconnected network of lysosomal proteases rather than as an isolated enzyme. Cathepsins L and D are particularly relevant because they contribute substantially to lysosomal protein degradation and may process substrates that overlap partly with those of cathepsin B. Functional overlap, however, does not imply that these enzymes are completely interchangeable. Individual cathepsins differ in catalytic mechanism, substrate preference, intracellular distribution, and sensitivity to lysosomal conditions. Consequently, the effect of cathepsin B loss may range from a limited substrate-specific defect to broader lysosomal dysfunction, depending on the availability and activity of other hydrolases.

Early inhibitor studies in rat liver indicated that selective inhibition of cathepsin B had only a limited effect on bulk lysosomal protein degradation, whereas broader inhibition of cysteine proteases produced a substantially greater reduction in lysosomal proteolysis [39,40]. These findings indicate that cathepsin B contributes to lysosomal proteolysis but is not individually responsible for the complete degradation of most lysosomal cargo. Other cysteine and non-

cysteine proteases can maintain part of the degradative capacity when cathepsin B activity is reduced.

Studies examining autophagic proteolysis further demonstrate that cathepsin B and cathepsin L possess overlapping but distinct functions. In nutrient-deprived HeLa and Huh-7 cells, the cathepsin L inhibitor CAA0225 reduced long-lived protein degradation and strongly inhibited the turnover of LC3-II and GABARAP. The study reported a comparable effect of CA-074Me on long-lived protein degradation, but attribution of this treatment arm specifically to cathepsin B requires caution because the intracellular selectivity of CA-074Me is condition-dependent [13,41]. This suggests that two cathepsins may contribute similarly to overall cargo degradation while showing different efficiencies toward specific autophagic substrates. Interpretation of the cathepsin B arm of this study nevertheless requires caution because CA-074Me may inhibit additional cysteine cathepsins under cellular conditions.

More extensive lysosomal abnormalities are frequently observed when cathepsins B and L are inhibited or depleted together. Cermak et al. demonstrated that combined loss of these enzymes reduced lysosomal degradation of internalized epidermal growth factor receptor, increased LC3-II and lysosomal protein accumulation, altered intracellular cholesterol trafficking, and produced enlarged acidic compartments [42]. In INS-1 cells and pancreatic islets, separate pharmacological inhibition or knockdown of cathepsin B and cathepsin L also impaired autophagic processing. These interventions caused LC3-II accumulation, incomplete maturation of several lysosomal cathepsins, enlarged lysosomes, and increased apoptosis under glucotoxic stress [43]. Although these studies were not performed in cancer models, they provide mechanistic evidence that cysteine-cathepsin dysfunction can affect the wider lysosomal enzyme system rather than only the directly inhibited protease.

Compensatory changes have also been observed in cancer. In the pancreatic ductal adenocarcinoma model discussed in Section 2.5, genetic loss of cathepsin B was accompanied by increased levels of active cathepsin L [38]. This increase may preserve part of the lysosomal or extracellular proteolytic capacity and may help explain why CTSB deletion delayed, but did not completely prevent, tumor progression. Such chronic adaptation is particularly relevant to genetic knockout models because cells and tumors have sufficient time to modify the expression, maturation, or activity of other proteases.

Acute pharmacological inhibition and chronic genetic deletion may therefore produce different phenotypes. Acute inhibition may rapidly reduce catalytic activity before substantial compensation occurs, but the observed result can be confounded by incomplete intracellular target engagement or off-target inhibition of other cathepsins. Genetic deletion provides stronger target-specific evidence but may trigger adaptive changes during cell selection or tumor development. Similar findings from both approaches strengthen a causal conclusion, whereas divergent findings should prompt assessment of compensatory protease activity, inhibitor selectivity, and treatment duration.

Functional redundancy has direct implications for the interpretation of autophagy experiments. A weak effect after selective cathepsin B inhibition does not necessarily demonstrate that cathepsin B is irrelevant; remaining lysosomal proteases may preserve sufficient degradation. Conversely, a severe autophagy phenotype produced by a nominal cathepsin B inhibitor cannot automatically be attributed to CTSB if the compound simultaneously inhibits cathepsin L or disrupts lysosomal function more broadly. Robust studies should therefore measure the activity and maturation of several relevant cathepsins,

confirm CTSB target engagement, compare pharmacological and genetic interventions, and evaluate autophagic flux rather than relying solely on steady-state marker accumulation.

3. The Autophagy–Lysosome Pathway in Cancer

3.1 Core Molecular Machinery and Autophagosome Formation

Macroautophagy, hereafter referred to as autophagy, is a multistep lysosome-dependent pathway that captures intracellular material within double-membrane autophagosomes and delivers it for degradation. The pathway comprises initiation, phagophore nucleation, membrane expansion, cargo sequestration, autophagosome closure, maturation, fusion with lysosomes, and degradation of the enclosed material. These stages are functionally connected but experimentally distinguishable. Therefore, increased activity at an early stage does not necessarily indicate successful completion of the entire pathway [1].

Autophagy initiation is controlled partly by the cellular nutrient- and energy-sensing systems. Under nutrient-rich conditions, mechanistic target of rapamycin complex 1 (mTORC1) associates with and suppresses the ULK1–ATG13–FIP200 initiation complex. Nutrient deprivation or pharmacological mTORC1 inhibition reduces this inhibitory regulation and permits activation of the ULK1 complex [44]. During glucose or energy deprivation, AMP-activated protein kinase (AMPK) can additionally promote autophagy by directly phosphorylating ULK1. Conversely, active mTORC1 phosphorylates ULK1 at a site that disrupts its functional interaction with AMPK. ULK1 therefore acts as an integration point through which nutrient availability and cellular energy status regulate entry into the autophagic pathway [45].

Following activation, the ULK1 complex promotes recruitment and activation of an autophagy-specific class III phosphatidylinositol 3-kinase complex. This complex contains the lipid kinase VPS34, VPS15, Beclin 1, and ATG14 and produces phosphatidylinositol 3-phosphate at sites of phagophore formation. Beclin 1 can participate in molecularly distinct VPS34 complexes containing either ATG14 or UVRAG. The ATG14-containing complex is particularly associated with early autophagosome formation, whereas the existence of distinct Beclin 1 complexes demonstrates that Beclin 1 or VPS34 abundance alone cannot be interpreted as a specific measure of autophagy initiation [46].

Phosphatidylinositol 3-phosphate provides a membrane signal that recruits effector proteins, including members of the WIPI family. WIPI2 establishes a functional connection between phosphatidylinositol 3-phosphate production and the machinery responsible for LC3 conjugation. Dooley et al. demonstrated that WIPI2 directly recruits the ATG12–ATG5–ATG16L1 complex to developing autophagic membranes and that disruption of the WIPI2–ATG16L1 interaction impairs LC3 lipidation and autophagosome formation [47]. This step converts an early lipid signal into recruitment of the protein-conjugation machinery required for phagophore expansion.

Two ubiquitin-like conjugation systems contribute to autophagosomal membrane development. In the first system, ATG12 becomes covalently conjugated to ATG5 and associates with ATG16L1. The resulting ATG12–ATG5–ATG16L1 complex promotes the second conjugation pathway, in which members of the LC3/GABARAP protein family become attached to phosphatidylethanolamine within the developing membrane. The biochemical basis of this lipidation process was established by demonstrating that the yeast LC3 homologue Atg8 is covalently conjugated to phosphatidylethanolamine through a ubiquitin-like enzymatic system [48].

In mammalian cells, newly synthesized LC3 is processed by ATG4 to expose a C-terminal glycine, generating the cytosolic form commonly termed LC3-I. Subsequent conjugation to phosphatidylethanolamine produces membrane-associated LC3-II. Kabeya et al. demonstrated that processed LC3-II localizes to autophagosomal membranes, providing the experimental foundation for its widespread use as an autophagosome marker [49]. LC3 lipidation contributes to phagophore expansion, cargo recruitment, and membrane organization, but the amount of LC3-II detected at one time reflects the balance between its formation and subsequent lysosomal turnover rather than initiation alone [1,49].

During membrane expansion, cytoplasmic material can be incorporated non-selectively or recognized selectively through cargo receptors that connect designated substrates to LC3/GABARAP proteins. The expanding phagophore gradually surrounds the cargo and closes to form a double-membrane autophagosome. At this stage, the material has been sequestered but has not yet been degraded. The autophagosome must subsequently mature, acquire appropriate trafficking and fusion machinery, and fuse with a functional lysosomal compartment.

This distinction is essential in cancer research. Anticancer treatment may stimulate ULK1 activation, LC3 lipidation, or autophagosome formation while simultaneously damaging lysosomes or preventing autophagosome clearance. Such a treatment may therefore increase markers of early autophagy without increasing overall degradative flux. The molecular machinery described in this section establishes how autophagosomes are generated; the following sections will determine whether these structures mature and deliver their contents for effective lysosomal degradation [1].

3.2 Autophagosome Maturation and Fusion with Lysosomes

Autophagosome formation does not complete the autophagic pathway. After closure, the mature double-membrane autophagosome must be transported toward the endolysosomal system, tethered to an appropriate degradative compartment, and fused with a late endosome or lysosome. Fusion produces an autolysosome in which the inner autophagosomal membrane and enclosed cargo become accessible to lysosomal hydrolases. Failure at any of these stages can cause autophagosome accumulation without successful cargo degradation [1,50–53].

The small GTPase Rab7 contributes to the late maturation and intracellular trafficking of autophagic vacuoles. Jäger et al. demonstrated that interference with Rab7 function caused the accumulation of enlarged late autophagic structures and impaired their final maturation. Rab7 was not required for the initial formation of autophagosomes but was necessary for efficient progression toward a fully degradative compartment [50]. This distinction shows that early autophagic structures may form normally even when the late pathway is defective.

Before membrane fusion occurs, the autophagosome and lysosome must be brought into close physical proximity. The homotypic fusion and protein sorting complex, known as the HOPS complex, functions as an important tethering factor during this process. Jiang et al. demonstrated that HOPS interacts with the autophagosomal protein syntaxin 17 and is required for syntaxin 17-dependent autophagosome–lysosome fusion. Depletion of HOPS components produced an accumulation of unfused autophagosomes, indicating that membrane tethering is a distinct and essential stage preceding fusion [52].

PLEKHM1 provides an additional connection between autophagic membranes and the lysosomal fusion machinery. It interacts with LC3/GABARAP-family proteins on autophagosomes and associates with Rab7, HOPS components, and proteins involved in membrane fusion. Loss of PLEKHM1 impaired autophagosome–lysosome fusion and reduced the clearance of autophagic substrates. PLEKHM1 therefore functions as an adaptor that coordinates cargo-containing autophagosomes with the Rab7–HOPS-regulated lysosomal compartment [53].

The final membrane-fusion event is mediated principally by soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins, commonly termed SNAREs. Syntaxin 17 is recruited to completed autophagosomes and forms a trans-SNARE complex with cytosolic SNAP29 and the endosomal or lysosomal SNARE VAMP8. Depletion of any of these proteins causes autophagosome accumulation and reduces fusion with endosomes and lysosomes. The preferential recruitment of syntaxin 17 to closed autophagosomes also helps prevent premature fusion before cargo sequestration has been completed [51].

Successful fusion changes the physical identity of the compartment but does not by itself prove that degradation is functional. The resulting autolysosome must maintain an acidic lumen, contain appropriately matured hydrolases, preserve membrane integrity, and generate sufficient proteolytic activity to digest both the inner autophagosomal membrane and its cargo. A cell may therefore display normal autophagosome–lysosome fusion while still exhibiting defective autophagic flux because degradation inside the autolysosome is inefficient [1].

This distinction is particularly important when investigating cathepsin B. Cathepsin B is not a core component of the Rab7–HOPS–SNARE fusion machinery; its principal relevance begins after cargo has reached the degradative lysosomal compartment. Pharmacological reduction of cathepsin B activity may therefore permit autolysosome formation while impairing subsequent proteolysis. Alternatively, a compound may indirectly block fusion by disturbing lysosomal pH, membrane organization, trafficking, or the activity of multiple lysosomal proteins.

Accordingly, the detection of increased autophagosomes after cathepsin B targeting does not identify the precise location of the defect. Experiments must determine separately whether autophagosomes successfully colocalize and fuse with lysosomes and whether the resulting autolysosomes degrade their contents. This mechanistic separation between formation, fusion, and degradation provides the foundation for interpreting autophagic flux in the following sections.

3.3 Lysosomal Degradation and Autophagic Flux

Autophagic flux describes the dynamic progression of material through the complete autophagic pathway, from cargo sequestration and autophagosome formation to lysosomal delivery, degradation, and recycling. It therefore represents a rate-dependent process rather than the number of autophagosomes present at a single time point. Autophagosome abundance is determined by the balance between formation and clearance: an increased number may result from accelerated production, reduced lysosomal degradation, or simultaneous changes at both stages [1].

Following autophagosome–lysosome fusion, lysosomal acidification and hydrolytic activity permit degradation of the inner autophagosomal membrane together with its enclosed cargo. Proteins are progressively cleaved into peptides and amino acids, while additional macromolecules are processed into metabolites that can be returned to the cytosol. Efficient completion of this stage requires intact lysosomal membranes, appropriate luminal pH, mature hydrolases, and sufficient collective proteolytic capacity. Impairment of any of these conditions can reduce cargo clearance even when autophagosomes form and fuse normally.

LC3 is widely used to examine autophagosome abundance and turnover. Cytosolic LC3-I is conjugated to phosphatidylethanolamine to form LC3-II, which associates with both the outer and inner autophagosomal membranes [49]. After fusion, LC3-II associated with the inner membrane enters the autolysosomal lumen and is degraded together with the cargo. Tanida et al. demonstrated that lysosomal turnover of endogenous LC3-II, rather than its total cellular abundance alone, reflects autophagic activity [54]. Consequently, a high LC3-II level may indicate either increased autophagosome formation or reduced lysosomal clearance.

The selective autophagy receptor p62/SQSTM1 provides complementary information. p62 contains a ubiquitin-associated domain that recognizes ubiquitinated cargo and an LC3-interacting region that connects this cargo to LC3-family proteins on the developing autophagosome. Bjørkøy et al. showed that p62-containing protein aggregates are delivered to autophagic compartments and that inhibition of autophagy causes accumulation of p62 protein and p62-positive bodies [55]. Pankiv et al. subsequently demonstrated direct binding between p62 and LC3 and established the functional importance of this interaction for autophagic degradation of ubiquitinated protein aggregates [56].

Because p62 is itself an autophagic substrate, a reduction in p62 protein can be compatible with increased cargo degradation, whereas p62 accumulation can indicate impaired autophagic clearance. However, p62 is also regulated at the transcriptional and post-translational levels. Increased p62 protein may therefore reflect enhanced gene expression, altered protein synthesis, or changes in other degradation systems rather than autophagy blockade alone. Measurement of p62 mRNA can help determine whether an observed protein increase is caused partly by transcriptional induction [1].

The combined interpretation of LC3-II and p62 provides a useful preliminary framework. Increased LC3-II accompanied by reduced p62 is compatible with enhanced productive flux because autophagosome formation and cargo degradation may both be increased. In contrast, simultaneous accumulation of LC3-II and p62 is compatible with a late-stage defect in cargo clearance. Nevertheless, neither pattern is conclusive without direct flux assessment. Unchanged LC3-II levels also do not exclude increased autophagy, because accelerated formation and accelerated degradation may produce little net change in steady-state abundance [1,54].

The time point of measurement is similarly important. An early treatment response may increase autophagosome formation, whereas prolonged exposure may progressively impair lysosomal function. Analysis at only one time point can therefore conceal a transition from initially productive autophagy to later pathway blockade. Dose, treatment duration, basal autophagic activity, and the metabolic state of the cancer cells should all be considered when comparing experimental findings.

These principles are particularly important when evaluating cathepsin B targeting. Reduced lysosomal cathepsin B activity may decrease terminal proteolysis without preventing autophagosome formation or fusion. Such a defect could produce LC3-II and p62 accumulation even though early autophagy signaling remains active. However, because cathepsin B functions together with other lysosomal hydrolases, the magnitude of this effect depends on inhibitor selectivity, intracellular target engagement, residual protease activity, and compensation by other cathepsins.

Therefore, changes in LC3-II, p62, or autophagosome number should be described as changes in autophagy-related markers unless dynamic experiments demonstrate whether cargo turnover has increased or decreased. The methods used to make this distinction are discussed in the following section.

3.4 Experimental Assessment of Autophagic Flux and Lysosomal Function

Reliable assessment of autophagy requires dynamic measurements that distinguish autophagosome production from lysosomal clearance. Static measurements of LC3-II, p62, or fluorescent puncta provide information about the amount of autophagy-related material present at a particular time but do not independently reveal whether this material is being generated rapidly or accumulating because degradation has failed. Current methodological guidelines therefore recommend combining multiple complementary assays that examine different stages of the pathway [1,57].

One of the most widely used approaches is the LC3 turnover assay performed in the presence and absence of a late-stage autophagy inhibitor. Cells receiving the experimental treatment are compared under otherwise identical conditions with cells additionally exposed to an inhibitor that prevents lysosomal clearance. If LC3-II accumulates further after lysosomal inhibition, autophagosomes were still being produced and delivered toward the degradative compartment before the inhibitor was added. In contrast, little or no additional accumulation may indicate that the experimental treatment had already impaired late-stage flux. However, this result can also arise from insufficient inhibitor exposure, excessive treatment duration, reduced LC3 synthesis, or saturation of the assay. Interpretation should therefore be based on the difference between matched conditions rather than on the absolute amount of LC3-II in a single sample [1,57].

Bafilomycin A1 is commonly used in short-term flux experiments because it inhibits the vacuolar H⁺-ATPase and prevents normal lysosomal acidification. It can also interfere with autophagosome–lysosome fusion through a mechanism that is not explained solely by its effect on luminal pH [59]. Chloroquine and hydroxychloroquine accumulate within acidic compartments and are also used to disrupt late autophagy, but they should not be considered mechanistically identical to bafilomycin A1. In the cellular systems examined by Mauthe et al., chloroquine primarily reduced autophagosome–lysosome fusion rather than simply eliminating lysosomal acidity or proteolytic activity [60]. The selected inhibitor, concentration, treatment duration, and cancer-cell model can therefore influence the measured outcome.

Tandem fluorescent LC3 reporters provide spatial information about autophagosome maturation. The original mRFP–GFP–LC3 reporter uses the different sensitivities of GFP and mRFP to the acidic and degradative autolysosomal environment. Before fusion, autophagic structures generally retain both signals and appear yellow in merged images. After delivery to

an acidic compartment, the GFP signal is reduced while the red fluorescent signal remains detectable, producing red-only structures [58]. An increase in both yellow and red-only puncta may therefore be consistent with enhanced pathway activity, whereas preferential accumulation of yellow puncta can indicate defective maturation or fusion. Reporter expression level, lysosomal pH, imaging settings, and incomplete quenching of GFP must nevertheless be controlled before puncta are interpreted quantitatively.

A related quantitative reporter, GFP–LC3–RFP–LC3ΔG, was developed to provide an internal control for expression. Endogenous ATG4 proteases cleave the construct into equimolar components. GFP–LC3 enters the autophagic pathway and is progressively degraded, whereas RFP–LC3ΔG remains predominantly cytosolic and serves as an internal reference. A reduction in the GFP-to-RFP ratio indicates increased autophagic degradation, while relative preservation of GFP is compatible with reduced flux [61]. This method can improve quantitative comparison but, like other LC3-based reporters, does not independently identify the exact lysosomal defect.

Direct evaluation of cargo degradation provides additional evidence. Turnover of p62 and other autophagic substrates can be examined over time, preferably together with measurements of their mRNA expression. Degradation of radiolabeled or pulse-labeled long-lived proteins provides a broader measure of intracellular protein turnover, although lysosomal and non-lysosomal contributions must be separated experimentally. DQ-BSA consists of heavily quenched fluorescent albumin that emits fluorescence after proteolytic cleavage within endolysosomal compartments and can therefore be used to evaluate lysosomal proteolytic capacity. However, DQ-BSA reaches lysosomes principally through endocytosis and is not an autophagy-specific substrate. Reduced DQ-BSA fluorescence demonstrates impaired endolysosomal degradation but does not alone establish that autophagosome formation or fusion is defective [1]. DQ-BSA and long-lived protein degradation have been used together to confirm reduced lysosomal proteolysis in treated breast cancer cells.

Measurements of lysosomal condition are necessary when the investigated target is cathepsin B. LysoTracker staining provides information about the abundance and distribution of acidic compartments, whereas pH-sensitive probes can more directly estimate lysosomal acidity. Colocalization of LC3 with lysosomal proteins such as LAMP1 can support delivery or proximity but cannot prove successful membrane fusion or cargo degradation. Lysosomal membrane damage can be investigated by galectin puncta formation, cytosolic redistribution of lysosomal enzymes, or biochemical separation of cytosolic and lysosomal fractions. These measurements help distinguish defective acidification, impaired fusion, reduced proteolysis, and lysosomal membrane permeabilization [1].

Cathepsin B should be assessed at the levels of protein abundance, maturation, localization, and catalytic activity. Immunoblotting may distinguish procathepsin B from its mature forms, but it does not demonstrate that the detected enzyme remains active. Fluorogenic substrates or activity-based probes can measure catalytic activity, although substrate and probe selectivity must be established. A convincing pharmacological study should additionally determine whether the compound reaches the relevant lysosomal or extracellular CTSB pool and whether other cysteine cathepsins are inhibited simultaneously.

Electron microscopy can identify double-membrane autophagosomes and degradative autolysosomal structures with high morphological resolution. Nevertheless, an increased

number of autophagic vacuoles in a static electron micrograph cannot distinguish increased formation from reduced clearance. Similarly, increased LC3 puncta or autophagosome number should not be described as increased flux unless degradation has been evaluated dynamically [1].

For studies examining pharmacological targeting of cathepsin B, the strongest experimental design combines several levels of evidence: confirmation of CTSB target engagement; comparison of LC3-II turnover with and without a late-stage inhibitor; measurement of p62 or another cargo substrate; assessment of lysosomal acidity, membrane integrity, and proteolytic capacity; and evaluation of the final cellular outcome. Genetic CTSB depletion or rescue can provide complementary evidence, although chronic genetic manipulation may produce compensatory changes that differ from acute pharmacological inhibition.

A claim that cathepsin B targeting induces autophagy is therefore justified only when increased pathway initiation is accompanied by evidence of continued lysosomal delivery and cargo degradation. When autophagosomes and LC3-II accumulate without additional turnover, while p62 or undegraded cargo also accumulates, the more appropriate interpretation is impaired late-stage flux. This methodological distinction will be used as a central criterion when evaluating the original cancer studies in Chapter 5.

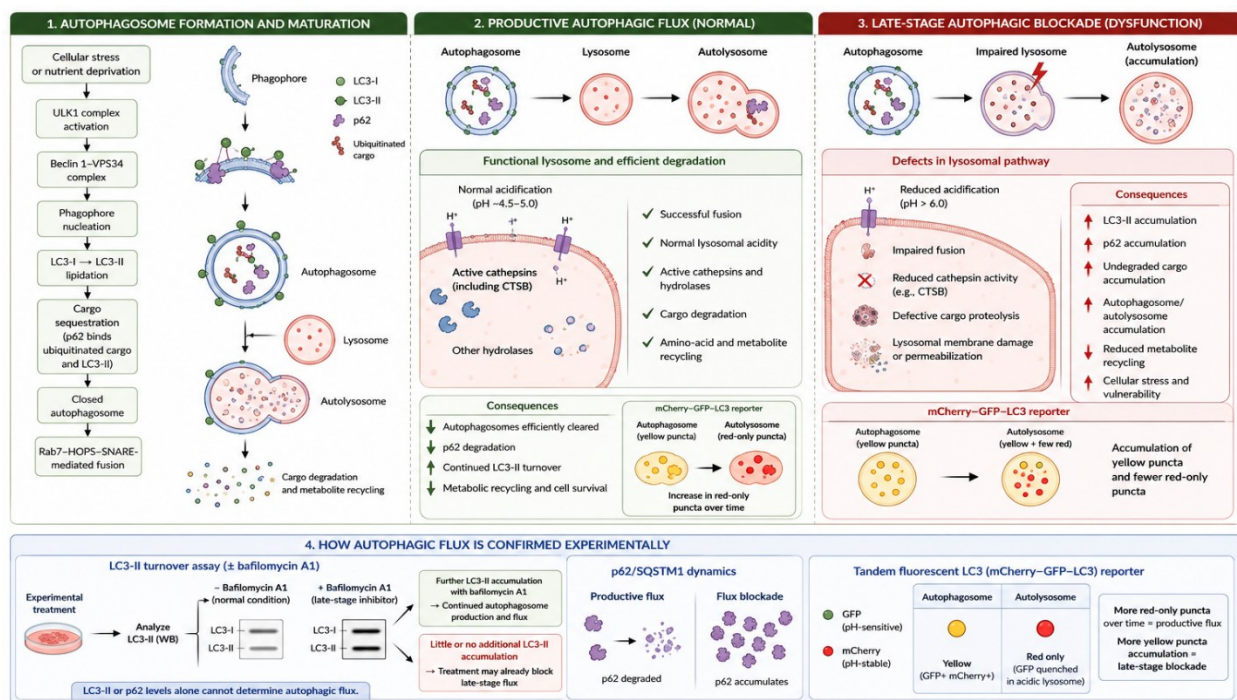


Figure 3. Autophagy–lysosome pathway and distinction between productive autophagic flux and late-stage blockade.

Autophagy proceeds through phagophore nucleation, LC3 lipidation, cargo sequestration, autophagosome closure, fusion with lysosomes, and degradation within autolysosomes. Productive flux requires functional fusion, lysosomal acidity, mature hydrolases, and effective cargo proteolysis. Under these conditions, LC3-II and p62 undergo lysosomal turnover and degradation products are recycled. In contrast, defective fusion, acidification, cathepsin activity, or lysosomal integrity can cause LC3-II, p62, and undegraded cargo to accumulate despite continued autophagosome formation. Dynamic flux assays, including LC3-II turnover in the presence and absence of a late-stage inhibitor and tandem fluorescent LC3 reporters, are required to distinguish increased productive flux from late-stage blockade. Conceptual synthesis based on references [1,44–61].

Created by the author with the assistance of artificial intelligence.

Figure 3. Autophagy–lysosome pathway and distinction between productive autophagic flux and late-stage blockade

3.5 Context-Dependent Functional Roles of Autophagy in Cancer

Autophagy has context-dependent and apparently opposing functions in cancer. Its biological effect varies according to the stage of tumor development, oncogenic background, tissue type, metabolic environment, treatment, and degree of pathway activity. Autophagy may

suppress early tumor development by limiting the accumulation of damaged organelles and stress-associated macromolecules, while established tumors may use the same pathway to maintain metabolism and survive environmental or therapeutic stress.

Evidence for a tumor-suppressive function was obtained from mice with systemic mosaic deletion of Atg5 and mice with liver-specific deletion of Atg7. These animals developed multiple benign liver tumors containing swollen mitochondria, accumulated p62, oxidative stress, and genomic-damage responses. Simultaneous deletion of p62 reduced tumor size in the Atg7-deficient livers, indicating that defective cargo clearance and p62 accumulation contributed to tumor formation [62]. These findings support a protective role for basal autophagy against tumor initiation, although the observed phenotype was strongly tissue-dependent and cannot be generalized to every organ or cancer type.

In contrast, several established tumors depend on autophagy for continued growth. Oncogenic RAS-driven cells and pancreatic cancer cells were shown to require autophagy for mitochondrial function, metabolic adaptation, and survival [3,4]. In genetically engineered lung-cancer models, autophagy loss disrupted metabolic homeostasis and impaired the maintenance of established tumors [5]. Strohecker et al. additionally demonstrated that Atg7 supported the growth of BRAF^{V600E}-driven lung tumors by maintaining mitochondrial function and glutamine metabolism [63]. These studies indicate that inhibition of autophagy may become therapeutically advantageous when an established tumor is metabolically dependent on lysosomal recycling.

The biological role of therapy-associated autophagy cannot be determined from LC3-II, p62, or autophagosome number alone. For analytical purposes, autophagy can be described as cytoprotective, cytotoxic, cytostatic, or nonprotective. These terms refer to the functional consequence of autophagy for the treated cancer cell rather than to the magnitude of marker changes. Classification therefore requires experimental interference with the autophagic pathway followed by measurement of survival, death, proliferation, or recovery.

Cytoprotective autophagy supports cancer-cell survival. It is demonstrated when pharmacological or genetic suppression of autophagy increases treatment-induced death, reduces clonogenic recovery, or impairs tumor growth. This function is consistent with studies showing that autophagy preserves mitochondrial metabolism and supplies recycled substrates to stressed RAS-, pancreatic-, BRAF-, and KRAS-driven tumors [3–5,63]. In this setting, autophagy inhibition may sensitize malignant cells to treatment, although benefit depends on effective pathway inhibition and tumor dependence on autophagic recycling.

Cytotoxic autophagy contributes functionally to cancer-cell killing. Evidence for this classification requires that interruption of essential autophagy machinery reduces, rather than increases, treatment-induced death. In VHL-deficient renal carcinoma cells, STF-62247 induced autophagy-associated cytotoxicity and preferentially reduced the growth of VHL-deficient tumors. Suppression of ATG5, ATG7, or ATG9 reduced cellular sensitivity to the compound, supporting a functional contribution of the autophagic machinery to cell killing [64]. Nevertheless, the presence of extensive autophagic structures during cell death is insufficient to establish cytotoxic autophagy unless inhibition of the pathway reproducibly protects the cells.

Cytostatic autophagy restricts proliferation or proliferative recovery without being the principal cause of immediate cell death. Sharma et al. reported that vitamin D or its analogue

EB1089 converted radiation-induced autophagy in non-small-cell lung cancer cells into a response associated with prolonged growth arrest and reduced clonogenic recovery. Pharmacological or genetic interference with autophagy reversed this sensitizing effect, supporting classification of the response as cytostatic rather than directly cytotoxic [65]. This distinction is important because short-term viability assays may underestimate therapeutic effects that primarily prevent long-term tumor-cell regrowth.

Nonprotective autophagy occurs when treatment changes autophagic activity but experimental suppression of the pathway does not substantially alter cell survival, treatment sensitivity, or proliferative recovery. In this situation, autophagy is a measurable stress response but is not a major determinant of the final cellular outcome. A lack of effect after autophagy inhibition must nevertheless be interpreted cautiously because incomplete pathway inhibition or compensatory mechanisms may falsely suggest that autophagy is functionally irrelevant.

The same treatment may produce different functional forms of autophagy depending on drug concentration, treatment duration, genetic background, and combination therapy. An initially cytoprotective response may later become ineffective because lysosomal degradation fails, while a second treatment may convert survival-promoting autophagy into cytostatic or death-associated autophagy. Functional classification should therefore be made separately for each experimental condition rather than assigned permanently to a particular cancer type or compound.

This framework is directly relevant to cathepsin B targeting. Accumulation of LC3-II after CTSB inhibition may reflect a protective response to lysosomal stress, a cytotoxic failure of cargo clearance, a growth-limiting response, or an epiphenomenon that does not determine cell fate. Correct interpretation requires three complementary levels of evidence: dynamic assessment of autophagic flux, confirmation that cathepsin B or lysosomal function was altered, and functional testing of whether autophagy changes cancer-cell survival, death, or proliferative recovery.

3.6 Lysosomal Dependency, Metabolic Adaptation, and Therapy Resistance

Established cancer cells may become dependent not only on autophagosome formation but on the coordinated activity of the entire autophagy–lysosome system. This dependency can provide amino acids and other metabolites, preserve mitochondrial quality, remove damaged cellular components, and help malignant cells tolerate nutrient deprivation, hypoxia, and therapeutic stress. It is particularly evident in tumors with high basal autophagic activity or oncogenic programs that generate substantial metabolic demand [3–5,63,66].

Pancreatic ductal adenocarcinoma provides one of the clearest examples of enhanced autophagy–lysosome dependency. Perera et al. demonstrated that pancreatic cancer cells activate a coordinated transcriptional program involving the MiT/TFE transcription-factor family. MITF, TFE3, and TFEB displayed increased nuclear activity and promoted the expression of genes involved in lysosome biogenesis, autophagy, and nutrient scavenging. Disruption of this program reduced intracellular amino-acid availability, impaired mitochondrial metabolism, and suppressed tumor growth, indicating that lysosomal activity supported the metabolic state of these cancer cells [66].

Autophagy–lysosome activity can also protect cancer cells from treatment-induced stress. In BRAF-mutant melanoma, Ma et al. showed that BRAF inhibition induced endoplasmic-reticulum stress and an autophagic response. Cells displaying stronger treatment-induced autophagy were less sensitive to the BRAF inhibitor, whereas pharmacological or genetic suppression of autophagy increased treatment-induced cell death. The findings provided functional evidence that autophagy contributed to acquired or adaptive treatment resistance rather than merely accompanying the therapeutic response [67].

Similar observations were obtained in RAS-driven cancers. Kinsey et al. demonstrated that inhibition of the RAF–MEK–ERK pathway increased autophagic activity and generated a protective dependency that could be therapeutically exploited by combining pathway inhibition with autophagy suppression [68]. In parallel, Bryant et al. showed that ERK inhibition increased autophagic flux in pancreatic cancer models and that simultaneous interference with ERK signaling and autophagy produced stronger antitumor effects than either intervention alone. These effects were evaluated using pharmacological and genetic approaches and were reproduced in several preclinical tumor models [69].

The protective effects of the autophagy–lysosome pathway are not restricted to metabolic recycling. Yamamoto et al. demonstrated that pancreatic cancer cells can deliver major histocompatibility complex class I molecules to lysosomes for degradation. Autophagy inhibition increased surface MHC-I expression, improved antigen presentation, and enhanced antitumor immune responses in experimental models. Thus, elevated lysosomal degradation may help pancreatic tumors evade immune recognition in addition to supporting their intracellular metabolism [70].

These studies do not establish that all cancers are uniformly dependent on autophagy or lysosomal activity. Dependency can vary according to tumor genotype, tissue of origin, nutrient availability, immune environment, basal autophagic activity, and treatment. Furthermore, a pathway that is essential during acute therapeutic stress may be less important during untreated growth, while prolonged inhibition may permit the development of compensatory metabolic or proteolytic mechanisms.

It is also necessary to distinguish autophagy dependency from broader lysosomal dependency. Lysosomes degrade material received through autophagy, endocytosis, macropinocytosis, and other trafficking pathways. Pharmacological compounds that alter lysosomal pH, membrane stability, trafficking, or enzyme activity can therefore affect several processes simultaneously. Sensitization to a lysosome-targeting compound does not by itself prove that inhibition of canonical autophagy is solely responsible for the observed cell death [1,60,66].

This distinction is directly relevant to cathepsin B. Tumors with high lysosomal throughput may require extensive proteolytic capacity to complete cargo degradation and maintain nutrient recycling. In such tumors, inhibition of lysosomal cathepsin B could reduce the efficiency of terminal degradation and weaken an adaptive survival mechanism. However, cathepsin B constitutes only one component of the lysosomal hydrolase network. Its inhibition may produce a limited effect when other proteases compensate, or a stronger phenotype when CTSB performs a nonredundant role in the investigated tumor model.

The final effect of CTSB targeting must therefore be evaluated at several levels: inhibition of the relevant enzyme pool, alteration of collective lysosomal proteolysis, change in autophagic

flux, and modification of cancer-cell survival or treatment response. This framework connects the molecular description of autophagy in the present chapter with the pharmacological strategies and original CTSB-related cancer studies examined in the following chapters.

4. Pharmacological Strategies Involving Cathepsin B

4.1 Defining the Pharmacologically Relevant Cathepsin B Pool

Cathepsin B should not be regarded as a single uniform pharmacological target. The enzyme exists in different molecular forms and cellular compartments, including inactive procathepsin B within the secretory pathway, mature enzyme within lysosomes and autolysosomes, cell-surface-associated and extracellular enzyme, and cathepsin B released into the cytosol after lysosomal membrane permeabilization. The pharmacologically relevant CTSB pool is therefore the catalytically active fraction located in the compartment responsible for the biological process being investigated [18–22,30–33].

For the present thesis, the lysosomal pool is of primary importance because it has the most direct mechanistic relationship with autophagic degradation. To inhibit this pool, a compound must enter the cell, reach the endolysosomal system, remain chemically functional under lysosomal conditions, and engage mature active cathepsin B. Demonstration of reduced total CTSB expression or extracellular enzyme activity does not establish that lysosomal CTSB has been inhibited. Similarly, cellular uptake of an inhibitor does not prove that an effective concentration has reached the lysosomal lumen.

The functional consequences of targeting lysosomal cathepsin B may include reduced proteolysis of autophagic cargo, altered autolysosomal degradation, and decreased metabolic recycling. In pancreatic ductal adenocarcinoma, preservation of cathepsin B activity was associated with increased autophagic flux and glycolytic metabolism [11]. This finding provides a rationale for investigating whether inhibition of the lysosomal CTSB pool can weaken a tumor-supportive recycling pathway. However, the result cannot be generalized without considering residual activity of other lysosomal proteases and the degree to which the investigated tumor depends on autophagic degradation.

A distinct pharmacological situation occurs when cathepsin B is released from lysosomes into the cytosol. In this location, the enzyme may participate in treatment-induced death signaling or cleavage of cytosolic substrates. Cytosolic redistribution of cathepsin B contributed to BCR–ABL degradation after imatinib-induced lysosomal membrane permeabilization in chronic myelogenous leukemia cells [32]. Lysosomal cathepsin release also contributed to the killing of treatment-resistant glioma cells, while cathepsin B inhibition reduced apoptosis in SPARC-overexpressing primitive neuroectodermal tumor cells [12,33]. In these contexts, pharmacological inhibition may protect cancer cells by interrupting a death-promoting pathway rather than increasing therapeutic cytotoxicity.

The extracellular and cell-surface-associated pools are pharmacologically relevant mainly to tumor invasion and matrix degradation. In inflammatory breast cancer cells, the poorly permeable inhibitor CA-074 reduced pericellular collagen degradation and invasion [30]. CA-074 administration also reduced bone metastasis in an experimental breast cancer model [31]. These findings support extracellular or pericellular CTSB as an anticancer target, but they do not establish inhibition of lysosomal CTSB or an effect on autophagic flux. An inhibitor can therefore produce an antimetastatic effect without substantially altering intracellular lysosomal degradation.

The cellular source of cathepsin B represents another important variable. Tumor cells, macrophages, and other stromal cells may contribute different CTSB pools within the tumor

microenvironment. Genetic experiments demonstrated contributions from both tumor-cell-derived and host-derived cathepsin B in mammary cancer progression, whereas forced CTSB overexpression in cancer cells produced stronger effects than comparable overexpression in stromal compartments in another experimental model [36,37]. A systemically administered inhibitor may consequently affect malignant cells and nonmalignant host cells simultaneously, making the observed tumor response a combined effect rather than a cancer-cell-specific outcome.

Pharmacological strategies involving cathepsin B can be divided into three principal categories. The first comprises direct inhibitors intended to reduce CTSB catalytic activity. The second includes compounds that indirectly alter CTSB expression, maturation, trafficking, lysosomal environment, or release without necessarily binding the enzyme directly. The third exploits CTSB activity as an enzymatic trigger for prodrug activation or controlled payload release. The third category does not represent CTSB inhibition and must therefore be analyzed separately from interventions intended to suppress enzyme function.

A study should consequently define which CTSB pool is expected to be affected and provide evidence that this pool was actually engaged. Relevant evidence may include measurement of mature enzyme forms, compartment-specific catalytic activity, intracellular localization, inhibitor accumulation, activity-based probe labeling, or functional rescue experiments. Without such information, the phrase “cathepsin B targeting” remains too broad to identify the mechanism responsible for changes in invasion, autophagy, metabolism, or cell death.

Throughout this thesis, pharmacological findings will therefore be interpreted according to four questions: whether the intervention directly or indirectly affects cathepsin B; whether it reaches the relevant cellular compartment; whether CTSB inhibition is selective and experimentally confirmed; and whether the measured biological outcome corresponds to lysosomal, cytosolic, or extracellular enzyme activity. This compartment-based framework is necessary for distinguishing genuine effects of lysosomal CTSB targeting on autophagy from unrelated consequences involving other cathepsin B pools.

4.2 Direct Cathepsin B Inhibitors: Mechanisms and Experimental Scope

Direct cathepsin B inhibitors reduce enzymatic activity through interaction with the catalytic cleft of the mature protease. Because catalysis depends on the active-site cysteine, many experimental inhibitors contain an electrophilic group capable of reacting with this residue. These compounds can be classified broadly as irreversible covalent inhibitors or reversible inhibitors. This distinction affects the duration of enzyme inhibition, the interpretation of washout experiments, and the risk that structurally related cysteine proteases will be inhibited simultaneously.

E-64 is a classical irreversible inhibitor containing an epoxysuccinyl reactive group. Barrett et al. demonstrated that E-64 and related compounds inhibit several cysteine proteases, including cathepsins B, H, and L. Inactivation occurs through covalent modification of the catalytic cysteine, producing sustained suppression of enzyme activity [71]. E-64 is therefore a valuable tool for demonstrating the involvement of cysteine proteases collectively, but it cannot assign a cellular phenotype specifically to cathepsin B.

The epoxysuccinyl scaffold was subsequently modified to increase affinity and selectivity for cathepsin B. CA-074 was designed to exploit structural characteristics of the CTSB substrate-

binding cleft and occluding loop. In the original biochemical and in vivo characterization, CA-074 displayed substantially greater selectivity for cathepsin B than for the other proteases examined [72]. However, selectivity demonstrated against purified enzymes does not automatically establish selective inhibition in intact cells, where membrane permeability, intracellular conversion, pH, reducing conditions, and inhibitor concentration can change pharmacological behavior. These limitations are examined specifically in Section 4.3.

Broad-spectrum direct inhibition may nevertheless have therapeutic value when several cysteine cathepsins cooperate in tumor progression. JPM-OEt is a cell-accessible epoxysuccinyl inhibitor that suppresses multiple active cysteine cathepsins rather than selectively targeting CTSB. In the RIP1-Tag2 model of pancreatic islet-cell tumorigenesis, JPM-OEt enhanced the effect of a chemotherapy regimen, produced significant tumor regression, and improved survival [73]. This study demonstrated that pharmacological inhibition of the cathepsin network can generate antitumor activity, but the result cannot be interpreted as evidence that cathepsin B alone was responsible.

A different strategy involves reversible inhibition. VBY-825 is a reversible multi-cathepsin inhibitor with high potency against cathepsins B, L, S, and V; its antitumor effects therefore do not constitute CTSB-specific pharmacological validation [74]. The compound therefore provided additional evidence that suppression of cysteine-cathepsin activity can impair tumor progression. Nevertheless, because several cathepsins were inhibited, the observed phenotype represented multi-cathepsin targeting rather than selective pharmacological validation of CTSB [74].

Irreversible and reversible inhibitors each present experimental advantages and limitations. Irreversible compounds can maintain enzyme suppression after the free inhibitor concentration decreases, but prolonged covalent inhibition may amplify off-target effects. Reversible compounds permit activity to recover when exposure is reduced, although their intracellular effectiveness depends continuously on free concentration, distribution, and target affinity. The chemical reversibility of an inhibitor therefore does not determine its selectivity; a reversible compound can still inhibit several related cathepsins.

The distinction between selective and broad inhibition is particularly important in autophagy experiments. Simultaneous inhibition of cathepsins B, L, and other lysosomal proteases may produce substantial accumulation of LC3-II, p62, and undegraded cargo by reducing collective lysosomal proteolysis. Such a phenotype cannot be described as a cathepsin B-specific effect without complementary evidence. In contrast, a highly selective CTSB inhibitor may produce a weaker lysosomal phenotype because other proteases preserve part of the degradative capacity.

Direct inhibition must also be distinguished from indirect pharmacological modulation. A compound that decreases CTSB expression, disrupts lysosomal acidification, prevents enzyme maturation, or induces lysosomal membrane permeabilization may reduce measurable CTSB activity without binding directly to its catalytic site. Such interventions can be biologically important, but they should not be classified as direct cathepsin B inhibitors unless enzyme-inhibitor interaction or catalytic target engagement has been demonstrated.

A rigorous evaluation of a direct inhibitor should therefore include biochemical potency against purified CTSB, comparison with other relevant cathepsins, confirmation of inhibition in intact cells, measurement of mature CTSB activity, and evidence that the compound

reaches the intended enzyme compartment. In autophagy studies, these measurements should be combined with dynamic flux assays and assessments of broader lysosomal function. Without these controls, an apparent CTSB-related phenotype may instead result from incomplete cellular access, broad cysteine-protease inhibition, or general lysosomal dysfunction.

4.3 CA-074 and CA-074Me: Cellular Access, Selectivity, and Interpretive Limitations

CA-074 and its methyl-ester derivative CA-074Me are among the most frequently used pharmacological tools for investigating cathepsin B. Although their names are often used interchangeably in experimental discussions, the two compounds differ substantially in membrane permeability, biochemical potency, intracellular conversion, and selectivity. These differences directly affect whether an observed phenotype can be attributed specifically to inhibition of lysosomal cathepsin B.

CA-074 is an epoxysuccinyl irreversible inhibitor developed to exploit structural characteristics of the cathepsin B active-site cleft and occluding loop. Its free C-terminal carboxyl group contributes to interactions that favour inhibition of cathepsin B over several related cysteine proteases. The original biochemical characterization demonstrated substantially greater inhibition of cathepsin B than of the other proteases examined, establishing CA-074 as a relatively selective CTSB inhibitor [72]. However, biochemical selectivity against purified enzymes does not independently demonstrate cellular target engagement.

CA-074 does not efficiently cross the plasma membrane by passive diffusion because of its charged and polar structure. It should therefore not automatically be assumed to inhibit intracellular CTSB during short cellular exposures. Nevertheless, limited passive permeability does not mean that CA-074 is exclusively extracellular. Montaser et al. demonstrated that prolonged exposure permitted fluid-phase endocytosis of CA-074 and subsequent delivery to lysosomes, where endogenous cathepsin B was inhibited while cathepsin L remained unaffected in the investigated murine fibroblasts [13]. The intracellular effectiveness of CA-074 consequently depends on concentration, incubation time, endocytic activity, and cell type.

CA-074Me was developed to improve cellular entry by esterifying the C-terminal carboxyl group of CA-074. Masking this charged group increases membrane permeability, allowing the compound to enter cells more readily. CA-074Me itself functions principally as a proinhibitor: after cellular uptake, intracellular esterases hydrolyse the methyl ester and generate the more potent CA-074 form. Buttle et al. demonstrated selective intracellular cathepsin B inactivation by CA-074 methyl ester in human gingival fibroblasts under the specific concentration and exposure conditions examined [75]. This study provided the original justification for the widespread use of CA-074Me in cell-based experiments.

Subsequent findings demonstrated that intracellular selectivity is not guaranteed. Montaser et al. reported that CA-074Me inhibited both cathepsin B and cathepsin L in living murine fibroblasts, whereas CA-074 showed greater selectivity for cathepsin B under their conditions [13]. Differences between this study and the earlier fibroblast experiment may reflect inhibitor concentration, exposure duration, cell type, esterase activity, intracellular distribution, and the methods used to measure enzyme activity. CA-074Me should therefore

not be described as a universally selective CTSB inhibitor simply because it is derived from CA-074.

The intracellular redox environment may further alter inhibitor behaviour. Steverding demonstrated that CA-074 and CA-074Me could inhibit cathepsin L in the presence of reducing thiol compounds and reported inhibition of cathepsin L in living HL-60 cells. These results indicate that even CA-074 may not remain completely CTSB-specific under every biochemical or cellular condition [77]. It is therefore more accurate to describe CA-074 as relatively selective under defined conditions rather than as an absolutely specific inhibitor.

Inhibitor potency is also strongly influenced by pH. Yoon et al. demonstrated that CA-074 inhibited cathepsin B with nanomolar potency at lysosomal pH and was more than 100-fold more potent at pH 4.6 than at the neutral pH of 7.2. The free C-terminal carboxyl group was required for this pH-dependent interaction. In contrast, unhydrolysed CA-074Me was a substantially weaker direct inhibitor and did not display the same pH-dependent potency [76]. These findings support preferential biochemical inhibition of the acidic lysosomal CTSB pool by CA-074 but also demonstrate that the activity of CA-074Me depends on its intracellular conversion.

The pH dependence of CA-074 has important consequences for compartment-specific interpretation. At the same free inhibitor concentration, lysosomal cathepsin B may be inhibited more efficiently than enzyme released into the neutral cytosol or located in a near-neutral extracellular environment. Nevertheless, the biological result also depends on whether the inhibitor reaches each compartment and remains present at a sufficient concentration. Potency measured in a purified acidic enzyme assay therefore cannot be transferred directly to intracellular, cytosolic, or extracellular conditions.

These limitations are particularly important in studies of autophagy. If CA-074Me treatment produces LC3-II and p62 accumulation, the result may be compatible with reduced lysosomal degradation. However, the phenotype cannot automatically be attributed to cathepsin B alone because CA-074Me may also inhibit cathepsin L or affect collective lysosomal proteolysis. Similarly, the absence of an effect after short CA-074 exposure may reflect insufficient intracellular delivery rather than biological irrelevance of lysosomal CTSB.

Reliable use of these inhibitors consequently requires direct confirmation of cellular target engagement. Studies should measure mature cathepsin B activity after treatment, evaluate cathepsin L and other relevant cysteine proteases, and distinguish extracellular from intracellular enzyme inhibition. Activity-based probes, genetic CTSB depletion or rescue, and comparison between CA-074 and CA-074Me can strengthen target attribution. Autophagy experiments should additionally include dynamic flux measurements and assessment of lysosomal acidity, proteolytic capacity, and membrane integrity.

CA-074 and CA-074Me remain useful experimental tools, but neither compound should be treated as self-validating evidence of CTSB-specific inhibition. Conclusions should be restricted to cathepsin B only when intracellular access, catalytic inhibition, and selectivity against related proteases have been experimentally demonstrated.

4.4 Activity-Based Probes and Verification of Cellular Target Engagement

Demonstrating that a compound inhibits purified cathepsin B is insufficient to establish pharmacological target engagement in intact cancer cells. Cellular activity depends on membrane permeability, intracellular distribution, lysosomal delivery, local pH, inhibitor stability, and competition with endogenous substrates. Functional chemical probes can help determine whether catalytically active cathepsin B is present in the relevant compartment and whether treatment has effectively inhibited it.

Activity-based probes assess protease function through activity-dependent covalent labeling. In probes designed for cysteine cathepsins, a recognition scaffold positions a moderately reactive electrophilic group near the catalytic cysteine, whereas an attached fluorophore, affinity label, or bioorthogonal handle enables detection or enrichment of the labeled enzymes. Because labeling requires an accessible and catalytically competent active site, these probes can distinguish active CTSB from inactive precursors and measurements of total protein abundance [78].

DCG-04 was developed from the E-64 scaffold as a biotinylated epoxide probe for profiling active papain-like cysteine proteases. It enabled covalent labeling, enrichment, and identification of active proteases in complex cellular samples and was used to compare cysteine-protease activity during cancer-cell progression [78]. However, DCG-04 reacts with several cysteine proteases and should therefore be considered a broad profiling tool rather than a CTSB-specific probe.

Fluorescent and quenched activity-based probes permit direct visualization of cysteine-cathepsin activity. BMV109 contains a phenoxymethyl-ketone electrophile and remains relatively nonfluorescent until it reacts with an active cysteine cathepsin. Its covalent labeling enables visualization in living cells and tumors and subsequent identification of labeled enzymes by electrophoretic analysis. Nevertheless, BMV109 reacts with several cysteine cathepsins and cannot independently assign a fluorescent signal specifically to cathepsin B [79].

Compartment-directed probes can provide additional information about the intracellular source of enzyme activity. Wiedner et al. developed a lysosome-targeted activity-based profiling approach that labeled principally cathepsins B and Z in living cells. The study demonstrated that chemical delivery to a defined organelle can restrict the group of enzymes detected and showed changes in lysosomal cathepsin activity during starvation-associated autophagy [80]. However, detection of both cathepsins B and Z illustrates that lysosomal localization alone does not guarantee enzyme-level selectivity.

More selective chemical tools have subsequently been developed. Bhuiyan et al. described KDA-1 as a cell-permeable, clickable, and tag-free activity-based probe designed for human cathepsin B. KDA-1 covalently labeled the catalytic Cys25 residue and detected active cathepsin B in proteomes obtained from living MDA-MB-231 breast cancer and HEK293 cells [81]. This approach provides a more direct method for distinguishing CTSB activity from the activity of closely related cathepsins. Nevertheless, validation in a limited number of cellular models does not establish universal selectivity or distribution across all tumor types and intracellular compartments.

Activity-based probes are particularly useful in competition experiments. Cancer cells can first be treated with a candidate inhibitor and then exposed to a probe that labels the remaining active enzymes. Reduced CTSB labeling compared with untreated controls provides evidence of cellular target engagement. Simultaneous analysis of cathepsins B, L, S, and other labeled proteases can additionally reveal whether the compound acts selectively or suppresses several cysteine cathepsins.

The interpretation of probe signals requires several controls. Because many activity-based probes label enzymes irreversibly, the probe itself acts as an inhibitor and may alter lysosomal proteolysis if used at high concentrations or for prolonged periods. Probe entry, intracellular accumulation, reporter fluorescence, and enzyme labeling can also differ between cell types. Absence of a signal may therefore reflect insufficient probe delivery rather than absence of active enzyme.

Activity-based probes must also be distinguished from fluorogenic substrate probes. A fluorogenic substrate generates a signal after enzymatic cleavage but normally does not remain covalently attached to the enzyme responsible for that cleavage. Similar substrate sequences may be processed by several lysosomal or extracellular proteases. Consequently, increased fluorescence from a nominally cathepsin B-activated substrate does not independently prove that CTSB was the only activating enzyme or that activation occurred within lysosomes.

For autophagy studies, the most informative design combines activity-based profiling with biochemical and functional measurements. An investigator should confirm inhibition of mature lysosomal CTSB, evaluate activity of related cathepsins, measure lysosomal proteolytic capacity, and perform dynamic autophagic-flux assays. Agreement between reduced CTSB probe labeling, impaired cargo degradation, and a reproducible cellular phenotype provides stronger mechanistic evidence than any individual measurement alone.

Activity-based probes are therefore not principally therapeutic agents in the context of this thesis. Their major value is experimental: they help determine whether a pharmacological compound reached the relevant enzyme pool, inhibited catalytically active CTSB, and retained selectivity within intact cells. Such confirmation is essential before changes in autophagy or cancer-cell fate are attributed specifically to cathepsin B.

4.5 Cathepsin B-Activatable Prodrugs and Responsive Drug-Delivery Systems

Cathepsin B-activatable therapeutic systems exploit proteolytic activity rather than attempting to inhibit it. In these approaches, an anticancer payload is connected to a carrier, targeting molecule, or inactive masking group through a peptide linker designed for cleavage within a cathepsin-rich compartment. Successful activation generally requires delivery of the construct to the tumor, cellular internalization when applicable, access to an endolysosomal compartment, enzymatic cleavage of the linker, and release of a pharmacologically active payload.

A typical design contains three functional components: a therapeutic payload, a protease-sensitive peptide linker, and a carrier or targeting unit. A self-immolative spacer may additionally be placed between the peptide and the drug to permit rapid liberation of the unmodified active compound after enzymatic cleavage. Frequently used peptide sequences include phenylalanine–lysine, valine–citrulline, and longer oligopeptides such as glycine–

phenylalanine–leucine–glycine. However, classification of these linkers as cathepsin B-sensitive does not establish that they are exclusively cleaved by CTSB within living cells.

Dubowchik et al. developed doxorubicin immunoconjugates containing phenylalanine–lysine or valine–citrulline linkers and a para-aminobenzyloxycarbonyl self-immolative spacer. The conjugates were designed to remain stable in plasma, become internalized after antibody binding, and release free doxorubicin following lysosomal proteolysis. Both linker systems supported enzymatic drug release and antigen-dependent cytotoxicity, demonstrating the feasibility of using lysosomal proteases as intracellular activation triggers [82].

The valine–citrulline strategy was subsequently applied to antibody–drug conjugates containing monomethyl auristatin E. Doronina et al. reported that antibody–valine–citrulline–MMAE conjugates displayed strong antigen-dependent cytotoxicity and produced regression of established tumor xenografts with a wider therapeutic index than comparable conjugates containing less stable linkers [83]. The study established lysosomally cleavable peptide linkers as a powerful drug-delivery strategy, although it did not prove that cathepsin B was the only protease capable of releasing the payload.

Later experiments directly challenged the assumption of CTSB exclusivity. Caculitan et al. evaluated valine–citrulline-containing antibody–drug conjugates in cells lacking cathepsin B and found that linker processing and cytotoxic activity remained substantially preserved. Other lysosomal cysteine proteases were able to process the linker in the absence of CTSB [84]. Consequently, the expression “cathepsin B-cleavable linker” should usually be understood as describing the biochemical design and initial enzyme characterization rather than demonstrating absolute enzyme specificity in living tumors.

The contribution of cathepsin B may nevertheless differ according to the carrier and linker architecture. In an engineered albumin–MMAE conjugate system, Bernardim et al. compared a protease-cleavable valine–citrulline linker with its noncleavable stereochemical counterpart and found that cathepsin B-dependent processing was required for efficient payload release and antitumor activity in that specific platform [85]. Together with the findings of Caculitan et al., This result does not contradict the findings of Caculitan et al.; rather, it demonstrates that CTSB dependence varies according to carrier design, payload chemistry, linker configuration, and the intracellular processing pathway [84,85].

Cathepsin B-responsive designs are not limited to antibody conjugates. Shim et al. developed a carrier-free system in which doxorubicin was conjugated to the cathepsin B-cleavable peptide phenylalanine–arginine–arginine–glycine. The resulting prodrug self-assembled into nanoparticles and produced stronger cytotoxicity in cathepsin B-overexpressing tumor cells while reducing toxicity toward the investigated normal cells. The nanoparticle formulation also improved tumor accumulation and antitumor activity in experimental models [86].

Responsive liposomal systems provide another approach. Lee et al. incorporated a cathepsin B-sensitive peptide–doxorubicin conjugate into liposomes. Exposure to cathepsin B promoted degradation of the peptide component and drug release, and the formulation produced anticancer activity in HepG2 liver cancer cells [87]. However, evidence from purified enzyme assays and one cellular model does not establish that CTSB alone would control payload release across different tumors or in vivo environments.

Several limitations affect the interpretation of these systems. Extracellular proteases may cause premature activation, while insufficient intracellular uptake or lysosomal delivery may prevent drug release. Heterogeneous CTSB activity within tumors can produce unequal activation, and other cathepsins may either compensate for low CTSB activity or reduce the enzyme specificity of the linker. Differences between human and animal plasma stability may additionally influence preclinical results.

Confirmation of CTSB-dependent activation therefore requires more than detection of payload release. Appropriate experiments should compare cleavable and noncleavable linkers, measure release in purified-enzyme and intact-cell systems, evaluate related lysosomal proteases, and use CTSB inhibition or genetic manipulation when possible. Colocalization of the carrier with lysosomes can support the proposed delivery route but does not independently identify the enzyme responsible for linker cleavage.

These strategies have an indirect relationship with autophagy. Their intended mechanism is payload activation or controlled drug release rather than suppression of lysosomal cathepsin B. Changes in LC3-II, p62, lysosomal integrity, or autophagic flux may arise from the released drug, cellular stress, nanoparticle accumulation, or damage to the endolysosomal system. Such findings should not be interpreted as consequences of CTSB inhibition unless the intervention has also been shown to reduce enzyme activity.

Cathepsin B-activatable prodrugs and responsive carriers therefore represent an important but mechanistically separate branch of CTSB-directed cancer pharmacology. They demonstrate how tumor-associated proteolytic activity can be exploited therapeutically, while the central question of this thesis—how reduction of lysosomal CTSB function affects autophagy—must be evaluated primarily through direct and indirect enzyme-modulation studies.

4.6 Pharmacological Versus Genetic Targeting and Causal Attribution

Pharmacological inhibition and genetic manipulation provide related but non-equivalent evidence about cathepsin B function. A pharmacological inhibitor primarily tests whether catalytic activity accessible to the compound is required during a defined period of treatment. Genetic knockdown or deletion reduces the amount of CTSB protein and may affect catalytic activity, enzyme maturation, intracellular localization, and potential noncatalytic functions. Agreement between both approaches strengthens causal attribution, whereas disagreement may reveal off-target drug effects, incomplete target engagement, compensatory adaptation, or differences between acute and chronic CTSB loss.

Pharmacological inhibition offers several experimental advantages. It can be initiated rapidly, applied at different concentrations, combined with anticancer treatments, and examined across multiple time points. It also resembles the type of intervention that would ultimately be required therapeutically. However, the result depends on inhibitor selectivity, cellular permeability, intracellular distribution, exposure duration, and access to the relevant CTSB pool. As demonstrated for CA-074 and CA-074Me, biochemical selectivity does not guarantee selective intracellular inhibition, and a negative result may reflect insufficient lysosomal delivery rather than biological independence from CTSB [13,72,75–77].

Genetic approaches include transient small-interfering RNA, stable short-hairpin RNA, gene knockout, and conditional gene deletion. These methods can provide greater target specificity than a small-molecule inhibitor, but they introduce different limitations. Knockdown may be incomplete, while complete deletion can permit clonal selection and long-term adaptation. The resulting phenotype may therefore differ from the immediate effect of inhibiting CTSB activity in an established cancer cell.

Compensation by related proteases is especially important during prolonged genetic manipulation. In pancreatic ductal adenocarcinoma models, CTSB deletion was accompanied by increased levels of active cathepsin L [38]. Such adaptation may preserve part of the lysosomal or extracellular proteolytic capacity and weaken the apparent effect of CTSB loss. Conversely, an acute pharmacological inhibitor may affect CTSB before compensatory changes develop, but may simultaneously inhibit cathepsin L or additional cysteine proteases.

Convergent pharmacological and genetic evidence was obtained in an experimental breast-cancer bone-metastasis model. RNA-interference-mediated reduction of CTSB decreased collagen degradation and metastatic progression, while treatment with CA-074 produced a related reduction in bone metastasis [31]. The agreement between two mechanistically different interventions provides stronger evidence for a functional CTSB contribution than either approach alone. Nevertheless, this study principally addressed extracellular matrix degradation and metastasis rather than lysosomal autophagic flux.

Genetic experiments can also challenge conclusions initially based on pharmacological or biochemical assumptions. Valine–citrulline linkers were frequently described as cathepsin B-cleavable, yet Caculitan et al. demonstrated that antibody–drug conjugate processing and cytotoxicity remained substantially preserved in CTSB-deficient cells [84]. This finding showed that other lysosomal proteases could compensate for CTSB absence and that cleavage by purified CTSB did not establish exclusive dependence in intact cells.

For autophagy-related studies, acute catalytic inhibition and chronic genetic depletion should not be expected to produce identical outcomes. Acute inhibition may rapidly decrease terminal lysosomal proteolysis while leaving CTSB protein and lysosomal organization initially intact. Chronic depletion may alter lysosomal biogenesis, maturation of other hydrolases, cellular metabolism, and protease compensation. The treatment duration and adaptation period must therefore be considered when comparing pharmacological and genetic results.

Rescue experiments provide an additional level of causal evidence. Re-expression of wild-type CTSB in depleted cells can determine whether the phenotype is reversible and specifically related to CTSB loss. Comparison with a catalytically inactive CTSB mutant can further distinguish dependence on enzymatic activity from effects associated with the presence, trafficking, or molecular interactions of the protein. Rescue should restore CTSB in the relevant compartment and at a level that does not create artificial overexpression effects.

The strongest experimental strategy combines several complementary elements: confirmation of catalytic target engagement in intact cells; assessment of related cathepsins; use of more than one inhibitor or chemical scaffold; genetic reduction of CTSB; and rescue with appropriately localized wild-type or catalytically inactive enzyme. In studies of autophagy, these controls must be accompanied by dynamic flux measurements, lysosomal-function assays, and evaluation of cancer-cell fate.

Among the strategies discussed in this chapter, interventions that directly inhibit lysosomal CTSB or specifically alter its maturation and lysosomal activity are the most relevant to the central autophagy question. Extracellular inhibitors are more directly relevant to invasion, while CTSB-activatable delivery systems exploit enzyme activity rather than suppressing autophagic degradation. Clear separation of these strategies is necessary before a biological outcome can be attributed to pharmacological targeting of lysosomal cathepsin B.

4.7 Therapeutic Limitations, Safety, and Translational Requirements

Direct inhibition of cathepsin B has demonstrated antitumor potential in several experimental models, but successful translation requires a therapeutic window in which pathological CTSB activity is suppressed without unacceptable disruption of physiological lysosomal functions. This challenge is complicated by the broad tissue distribution of cathepsin B, the existence of several functionally distinct enzyme pools, and partial redundancy with other cysteine cathepsins.

The severe phenotype of combined CTSB/CTSL deficiency demonstrates functional redundancy and indicates that broad disruption of the lysosomal cysteine-protease network may be substantially more toxic than loss of cathepsin B alone [88]. This result indicates that functional compensation can preserve essential lysosomal activity after loss of one protease but also warns that inhibitors affecting several related cathepsins may produce substantially greater toxicity. Congenital gene deletion, however, cannot be treated as proof that prolonged pharmacological inhibition is safe because developmental adaptation may reduce the apparent consequences of CTSB loss.

Cathepsin B also participates in immune and inflammatory responses. In macrophages, genetic depletion or pharmacological inhibition of cathepsin B reduced the processing and secretion of tumor necrosis factor- α by interfering with the trafficking of TNF-containing vesicles toward the plasma membrane [89]. In a *Leishmania major* infection model, CTSB-deficient mice displayed altered T-cell-dependent inflammatory responses and resolved lesions more rapidly than wild-type animals [90]. These findings do not demonstrate universal immune suppression after CTSB inhibition, but they show that systemic targeting can modify immune-cell function in ways that may influence antitumor immunity, infection responses, and treatment toxicity.

Compartment selectivity represents a central translational requirement. The extracellular and cell-surface-associated CTSB pools may promote matrix degradation and metastasis, whereas lysosomal CTSB contributes to intracellular degradation and metabolic recycling. A systemically distributed, cell-permeable inhibitor may suppress both pathological extracellular activity and physiological lysosomal activity. In contrast, an extracellularly restricted inhibitor may reduce invasion while producing little effect on autophagic degradation. The optimal inhibitor design therefore depends on whether the therapeutic objective is to suppress invasion, impair lysosomal metabolism, or interfere with another CTSB-dependent process.

Targeted delivery may improve this compartmental and tissue selectivity. Mikhaylov et al. developed a liposomal nanocarrier displaying a lipidated cathepsin B inhibitor and demonstrated preferential targeting and internalization by tumor cells and tumor-associated macrophages in a mammary cancer model [91]. This proof-of-principle strategy suggests that tumor-associated CTSB can be used to concentrate a therapeutic platform within malignant

tissue. However, nanoparticle accumulation, uptake by phagocytic cells, and distribution through the tumor vasculature may also contribute to apparent selectivity and must be separated from CTSB-dependent targeting.

Selection of suitable tumors requires measurement of catalytic activity rather than reliance on CTSB expression alone. Kramer et al. developed a highly selective inhibitory designed ankyrin repeat protein that enabled non-invasive visualization of tumor-associated cathepsin B in experimental models [92]. Such activity-directed tools could support patient stratification, assessment of tumor heterogeneity, and confirmation that the relevant CTSB pool is accessible before treatment. Nevertheless, imaging of extracellular or tumor-associated activity does not independently establish that lysosomal CTSB within cancer cells is pharmacologically reachable.

Pharmacokinetic and pharmacodynamic properties must also be matched to the intended mechanism. A lysosomal inhibitor must remain stable in circulation, enter the appropriate cells, accumulate within endolysosomal compartments, and retain sufficient potency under acidic conditions. Irreversible inhibitors may produce prolonged target occupation after systemic drug levels decline, but this property can amplify off-target effects. Reversible inhibitors may permit greater control over exposure but require sustained concentrations at the target compartment. Plasma stability, protein binding, metabolism, clearance, tissue distribution, and intracellular conversion must therefore be evaluated together with enzyme inhibition.

Tumor heterogeneity creates an additional limitation. CTSB activity may differ between cancer cells, macrophages, stromal cells, metastatic sites, and regions of the same tumor. A treatment directed against one enzyme pool may consequently inhibit only part of the tumor-associated activity. Furthermore, chronic CTSB suppression may select cells that depend less strongly on the enzyme or increase the activity of compensating proteases such as cathepsin L. Combination strategies should therefore be based on demonstrated tumor dependency rather than on CTSB overexpression alone.

The biological direction of the treatment effect must also be considered. Inhibition of lysosomal CTSB may weaken metabolic recycling and sensitize autophagy-dependent tumors. Conversely, it may prevent CTSB-mediated apoptosis or cleavage of oncogenic proteins when lysosomal membrane permeabilization forms part of the treatment response [12,32,33]. The same inhibitor could therefore be beneficial with one anticancer treatment but counterproductive with another. Combination therapy requires experimental confirmation that CTSB inhibition enhances rather than antagonizes the intended cell-death mechanism.

Most direct-inhibitor evidence discussed in this chapter is derived from cultured cells and animal tumor models [31,73,74]. Before clinical translation, candidate inhibitors should be evaluated in immunocompetent and patient-derived models, with repeated-dose assessment of tumor activity, lysosomal function in normal tissues, immune responses, organ toxicity, and recovery after treatment withdrawal. Studies should additionally establish pharmacodynamic biomarkers demonstrating inhibition of the intended CTSB pool at tolerated exposure levels.

The principal translational challenge is therefore not simply to produce a potent cathepsin B inhibitor. A clinically meaningful strategy must identify a CTSB-dependent tumor, deliver the inhibitor to the disease-relevant enzyme compartment, demonstrate selectivity and target engagement, preserve an acceptable level of physiological lysosomal function, and produce a

favorable effect on cancer-cell fate. These criteria provide the framework for evaluating the original studies connecting cathepsin B targeting with autophagy in Chapter 5.

Figure 4. Pharmacological strategies involving cathepsin B and their distinct mechanisms

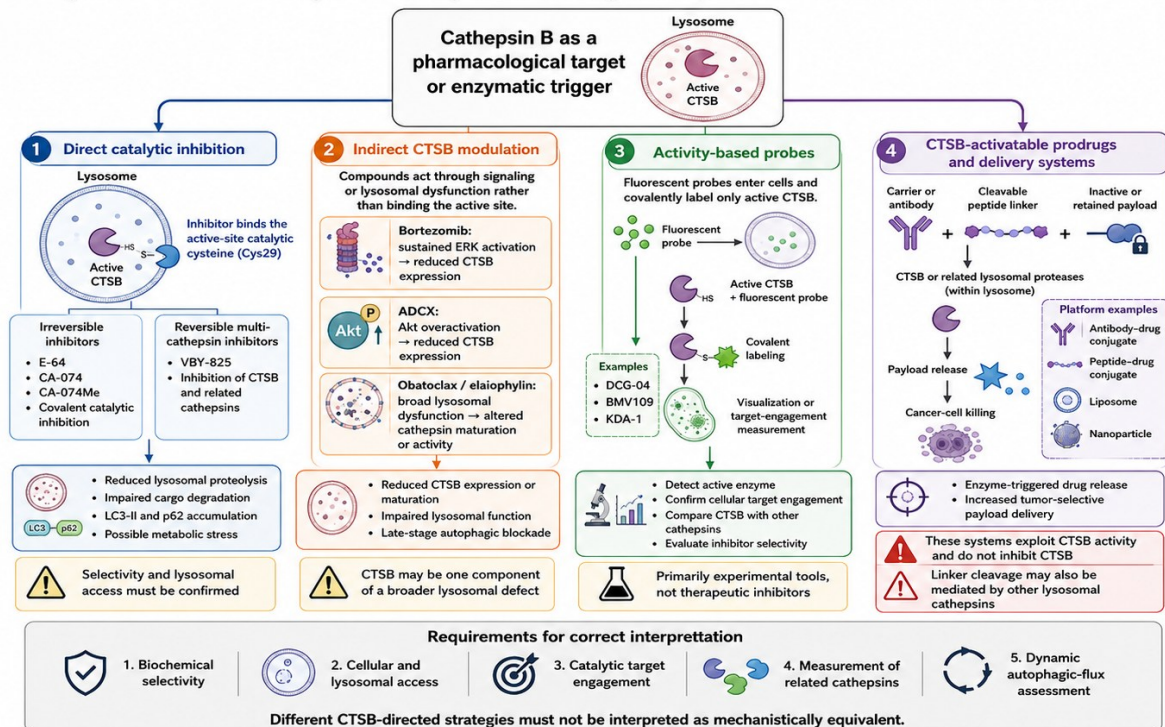


Figure 4. Pharmacological strategies involving cathepsin B and their distinct mechanisms. Direct inhibitors reduce cathepsin B catalytic activity through interaction with the active-site cleft, although intracellular access and selectivity against related cysteine cathepsins must be confirmed. Indirect pharmacological interventions alter CTSB expression, maturation, trafficking, activity, or the lysosomal environment without necessarily binding the enzyme directly. Activity-based probes detect catalytically active CTSB and can verify cellular target engagement and inhibitor selectivity. In contrast, CTSB-activatable prodrugs and responsive delivery systems exploit lysosomal proteolytic activity to release therapeutic payloads and do not represent CTSB inhibition. These strategies are mechanistically distinct and must be evaluated separately when interpreting effects on autophagy and cancer-cell fate. Conceptual synthesis based on references [9,10,13,71–87,94–98]. Created by the author with the assistance of artificial intelligence.

Figure 4. Pharmacological strategies involving cathepsin B and their distinct mechanisms

5. Experimental Evidence Linking Cathepsin B Targeting to Autophagy in Cancer Cells

5.1 Direct CTSB Inhibition and Evidence for Late-Stage Autophagy Blockade

The most direct approach for determining whether cathepsin B contributes to autophagic degradation is to inhibit its catalytic activity and subsequently measure dynamic autophagic flux. If early autophagosome formation continues while lysosomal cargo clearance decreases, LC3-II, p62, and undegraded material may accumulate. Such a result would support a late-stage degradative defect rather than increased productive autophagy. However, the available evidence remains limited because many studies use inhibitors with incomplete cellular selectivity or compounds that affect several lysosomal targets simultaneously.

Mechanistic evidence was provided by Lamore and Wondrak using human Hs27 skin fibroblasts. Ultraviolet A exposure decreased cathepsin B activity and produced lysosomal expansion together with increased LC3-II, p62, Beclin 1, and LAMP1. Pharmacological treatment with CA-074Me reproduced several of these alterations, leading the authors to propose that cathepsin B inactivation contributed to impaired autophagic–lysosomal processing [93]. Although this study supports a functional connection between cathepsin B activity and cargo clearance, it was not conducted in malignant cells and therefore cannot independently establish the therapeutic consequences of CTSB inhibition in cancer.

Interpretation of the CA-074Me experiment also requires consideration of inhibitor selectivity. As discussed in Section 4.3, CA-074Me may inhibit cathepsin L and other cysteine proteases under some cellular conditions [13,77]. The observed phenotype may consequently reflect reduced collective lysosomal proteolysis rather than exclusive inhibition of cathepsin B. Nevertheless, the study demonstrates that pharmacological suppression of a CTSB-containing lysosomal protease system can produce an autophagosome-clearance phenotype.

More direct cancer-related evidence was obtained with oblongifolin C, a natural compound initially identified through screening for autophagy modulators. In HeLa and additional cancer-cell models, oblongifolin C increased autophagosome abundance and LC3-II while preventing efficient degradation of p62. Colocalization experiments indicated impaired autophagosome–lysosome fusion, and lysosomal assays demonstrated altered acidification, reduced proteolytic activity, and decreased expression of lysosomal cathepsins. Treatment sensitized nutrient-deprived cancer cells to caspase-3-dependent apoptosis and produced compatible autophagy-marker and cell-death changes in a HeLa xenograft model [94]. These results provide substantial evidence that the compound causes late-stage autophagic blockade rather than productive pathway induction.

Oblongifolin C cannot, however, be regarded as a selective cathepsin B inhibitor. The original study demonstrated effects on lysosomal acidification, autophagosome–lysosome fusion, proteolytic activity, and the expression of several lysosomal cathepsins [94]. Any of these changes could contribute to reduced flux. Therefore, the study provides strong evidence for lysosomal autophagy blockade but only indirect evidence that CTSB inhibition is the principal initiating mechanism.

A later chemical-biology study provided evidence of a potential direct interaction between oblongifolin C and purified cathepsin B. Han et al. reported binding in surface-plasmon-

resonance and isothermal-titration-calorimetry experiments and inhibition of catalytic activity with an in vitro IC_{50} of approximately 31.9 μ M, while also identifying HSPA8 and additional proteins as possible pharmacological targets [95]. However, the compound also interacted with HSPA8 and affected additional cellular pathways. Moreover, the study did not demonstrate that selective restoration of CTSB activity reversed the autophagic-flux phenotype. The findings therefore support CTSB as one pharmacological target of oblongifolin C but do not establish it as the exclusive mediator of lysosomal dysfunction.

Together, these studies support the mechanistic principle that reduced cathepsin B-containing lysosomal proteolytic activity can be associated with LC3-II and p62 accumulation and impaired terminal autophagic degradation. The cancer evidence is strongest for the existence of a late-stage lysosomal blockade, whereas the evidence attributing that blockade specifically and exclusively to CTSB remains less conclusive.

A definitive demonstration of CTSB-specific autophagy blockade would require a selective intracellular inhibitor with confirmed lysosomal target engagement, measurement of related cathepsins, dynamic flux assays, and genetic rescue with active CTSB. None of the studies discussed in this section fully combines all these criteria. Accordingly, they should be interpreted as evidence supporting a CTSB-linked lysosomal mechanism rather than as proof that inhibition of cathepsin B alone is sufficient to block autophagic flux in every cancer model.

5.2 Indirect Pharmacological Suppression of CTSB and Lysosomal Dysfunction

Several anticancer compounds reduce cathepsin B expression, maturation, or activity without directly inhibiting its catalytic site. These interventions are relevant because they may impair terminal autophagic degradation, but their effects are usually broader than selective CTSB inhibition. The compound may simultaneously alter signaling pathways, lysosomal acidification, membrane integrity, autophagosome–lysosome fusion, or the activity of several lysosomal hydrolases. Consequently, these studies provide evidence for a CTSB-associated lysosomal mechanism but require careful evaluation before causality is attributed specifically to cathepsin B.

Bortezomib provides one of the strongest examples of indirect CTSB suppression in cancer cells. In TOV112D and TOV21G ovarian cancer cells, bortezomib increased LC3-II and p62, indicating autophagosome accumulation without efficient cargo degradation. The treatment decreased CTSB protein and mRNA expression through sustained ERK phosphorylation. Pharmacological inhibition of ERK restored CTSB levels and reduced p62 accumulation, whereas expression of constitutively active ERK decreased CTSB. These findings positioned CTSB downstream of ERK signaling rather than identifying it as a direct molecular target of bortezomib [9].

Importantly, forced CTSB expression partially restored p62 degradation, increased LysoTracker-associated signals, and protected ovarian cancer cells from bortezomib-induced loss of viability. This rescue experiment provides stronger causal evidence than the observation of reduced CTSB expression alone. Nevertheless, bortezomib also reduced other cathepsins, including cathepsin D, indicating that the lysosomal phenotype was not necessarily mediated exclusively by CTSB [9].

The therapeutic relevance of this mechanism was examined in combination with cisplatin. Cisplatin alone increased LC3-II while reducing p62, a pattern compatible with productive autophagic flux. Addition of bortezomib caused simultaneous accumulation of LC3-II and p62 and reduced cisplatin-associated cathepsin B and D expression. Combined treatment produced greater antitumor activity than bortezomib alone in an ovarian cancer mouse model. Tumor analysis showed increased ERK phosphorylation, reduced CTSB, and impaired p62 degradation [9]. These results support the interpretation that bortezomib blocked a treatment-induced protective recycling pathway and thereby enhanced cisplatin cytotoxicity.

However, the effect of bortezomib was not uniform across all examined ovarian cancer cell lines. Although p62 increased consistently, changes in ERK phosphorylation and CTSB expression varied in some models. The study also relied substantially on steady-state LC3-II and p62 measurements rather than a complete set of modern dynamic-flux assays. The ERK–CTSB mechanism should therefore be regarded as well supported in selected ovarian cancer models but not automatically generalizable to all solid tumors.

A related mechanism was reported for ADCX, a cycloartane triterpenoid examined in multidrug-resistant HepG2/ADM liver cancer cells. ADCX increased Akt activity, reduced lysosomal CTSB expression, impaired autophagic degradation, and promoted p62 and autophagosome accumulation. Interference with autophagic clearance increased ADCX-induced apoptotic cell death, suggesting that the autophagy–lysosome pathway contributed to the drug-resistant phenotype of these cells [10].

ADCX should not be classified as a direct cathepsin B inhibitor because direct binding to the CTSB catalytic site was not demonstrated. Its primary mechanistic interpretation was that Akt overactivation reduced CTSB expression and thereby weakened lysosomal degradation. Furthermore, because the study focused principally on one multidrug-resistant liver cancer model, the relevance of the Akt–CTSB pathway to non-resistant parental cells and other cancer types remains uncertain. The findings nevertheless provide cancer-specific evidence that signaling-mediated reduction of CTSB can contribute to late-stage autophagy impairment.

Broader lysosomal dysfunction was observed with obatoclax, a compound originally developed as an antagonist of anti-apoptotic BCL-2-family proteins. In cisplatin-sensitive and cisplatin-resistant esophageal cancer cells, obatoclax accumulated within lysosomes, caused lysosomal clustering, and produced simultaneous LC3-II and p62 accumulation. The treatment reduced active forms of cathepsins B, D, and L, demonstrating disruption of the lysosomal protease system rather than selective CTSB suppression [96].

Individual knockdown of CTSB, CTSD, or CTSL increased LC3-II, supporting a functional contribution of each protease to autophagic processing. In cell lines expressing detectable CTSB, its knockdown also reduced cell viability. However, CTSB was undetectable in some investigated esophageal cancer cells, whereas obatoclax still blocked autophagy through reductions in cathepsins D and L. This finding demonstrates that CTSB was relevant in selected cells but was not universally required for the lysosomal effect of obatoclax [96].

Elaiophylin provides another example of a compound that indirectly alters a CTSB-containing lysosomal system. In ovarian cancer cells, elaiophylin promoted autophagosome and LC3-II accumulation while preventing p62 degradation. It reduced lysosomal cathepsin activity, interfered with the maturation of cathepsins B and D, and destabilized lysosomes.

The compound displayed antitumor activity alone and increased cytotoxicity under hypoxic conditions or in combination with cisplatin [97]. These results support classification of elaiophylin as a late-stage autophagy inhibitor but not as a selective CTSB-targeted compound.

A subsequent study identified SHP2 as a direct molecular target of elaiophylin and showed that excessive SHP2–SOS1–MAPK activation induced endoplasmic-reticulum stress and paraptotic vacuolization. This process was accompanied by reduced CTSB and CTSD activity, lysosomal dysfunction, p62 accumulation, and autophagy inhibition in ovarian cancer cells and tumor models [98]. Inhibition of SHP2 partially restored cathepsin activity and reduced the lysosomal and autophagic abnormalities. These findings place cathepsin impairment downstream of a broader stress response rather than identifying CTSB as the initial pharmacological target.

Collectively, the four compounds demonstrate that indirect reduction of CTSB can accompany late-stage autophagic blockade and increased cancer-cell death. Bortezomib provides the strongest evidence for a CTSB-dependent component because CTSB restoration improved p62 degradation and cell survival. ADCX supports an Akt–CTSB pathway in multidrug-resistant hepatocellular cancer cells, whereas obatoclax and elaiophylin predominantly demonstrate multi-enzyme lysosomal dysfunction.

These studies also illustrate an important limitation of indirect targeting. When a compound alters several cathepsins, lysosomal pH, membrane stability, or intracellular signaling simultaneously, the resulting autophagic blockade cannot be attributed solely to CTSB. Strong causal attribution requires restoration or selective manipulation of CTSB together with dynamic flux analysis and measurement of other lysosomal enzymes. The available evidence therefore supports CTSB as one mechanistically relevant component of treatment-induced lysosomal dysfunction, but not as the exclusive mediator in most indirect pharmacological models.

5.3 Cathepsin B-Supported Autophagic Flux and Metabolic Adaptation

Evidence that preserved cathepsin B activity supports productive autophagic flux in cancer is more limited than the evidence connecting lysosomal dysfunction with late-stage pathway blockade. The strongest direct evidence currently comes from pancreatic ductal adenocarcinoma, in which lysosomal CTSB activity was linked mechanistically to autophagic degradation, metabolic substrate availability, and tumor-cell proliferation.

Jiang et al. identified coordinated enrichment of autophagy- and glycolysis-related signatures in pancreatic ductal adenocarcinoma and investigated cystatin B as a molecular connection between these processes. CSTB expression was increased in pancreatic cancer models and was associated with enhanced tumor-cell growth. Genetic depletion of CSTB reduced proliferation in cultured pancreatic cancer cells and patient-derived xenograft models, whereas CSTB overexpression promoted growth [11].

The study demonstrated that CSTB increased autophagic flux rather than merely increasing the steady-state abundance of autophagosomes. CSTB depletion reduced lysosomal cargo processing, while its overexpression promoted autophagic degradation. Importantly, bafilomycin A1 abolished the proliferative advantage produced by CSTB overexpression, indicating that completion of the autophagy–lysosome pathway was required for the growth-

promoting effect. These experiments functionally connected enhanced autophagic degradation with pancreatic cancer-cell proliferation [11].

Proteomic analysis identified cathepsin B as a CSTB-interacting protein.

Immunoprecipitation and immunofluorescence experiments further indicated that CSTB competed with cystatin C for binding to CTSB. In this model, increased CSTB reduced the inhibitory influence of cystatin C and sustained lysosomal CTSB proteolytic activity. Thus, despite belonging to the cystatin family, CSTB did not function simply as a conventional CTSB inhibitor under the investigated conditions [11].

Preservation of CTSB activity was linked to the metabolic consequences of productive autophagy. Increased autophagic degradation supplied intracellular substrates that supported glycolytic metabolism, whereas interference with autophagy or glycolysis reduced the difference in proliferation between CSTB-overexpressing and control cells. The findings therefore support a pathway in which CSTB sustains CTSB-mediated lysosomal proteolysis, promotes autophagic recycling, and supplies metabolites required for pancreatic cancer growth [11].

This study provides unusually strong evidence because it combines measurements of autophagic flux, lysosomal CTSB activity, protein-interaction experiments, genetic manipulation, metabolic analysis, and tumor-growth models. It also includes functional experiments showing that disruption of autophagic degradation eliminates the proliferative effect associated with CSTB. Among the currently available studies, it therefore provides the clearest demonstration that preservation of CTSB activity can support a tumor-promoting autophagy–metabolism axis.

Several limitations must nevertheless be considered. The investigated intervention primarily altered CSTB rather than directly targeting CTSB with a selective pharmacological compound. CSTB may possess additional functions unrelated to cathepsin B, and competition with cystatin C may affect the regulation of other cysteine proteases. Furthermore, the evidence was derived predominantly from pancreatic ductal adenocarcinoma, a tumor type characterized by unusually high autophagy–lysosome and metabolic activity. The same degree of CTSB dependency cannot be assumed in cancers with lower lysosomal throughput.

Complementary evidence was obtained in clear-cell renal-cell carcinoma treated with sunitinib. Giuliano et al. demonstrated that sunitinib accumulated within lysosomes, reduced CTSB activity, and produced incomplete autophagic flux. Lysosomal sequestration simultaneously reduced the amount of pharmacologically available drug and contributed to treatment resistance [99]. This study supports an association between reduced lysosomal CTSB activity and defective flux, but it does not demonstrate that CTSB inhibition alone caused sunitinib resistance because drug sequestration and broader lysosomal alterations occurred concurrently.

The renal-cell carcinoma findings also illustrate that impaired autophagic degradation does not necessarily increase treatment sensitivity. Late-stage flux blockade may promote cell death in one model but coexist with lysosomal drug sequestration and resistance in another. The final cellular effect therefore depends not only on cargo degradation but also on the pharmacokinetics of the anticancer compound, the extent of lysosomal damage, and the adaptive capacity of the tumor cells.

Collectively, the evidence indicates that active lysosomal cathepsin B can contribute to productive autophagic degradation and metabolic adaptation in selected tumors. The pancreatic cancer study provides direct functional support for this mechanism, whereas the renal-cell carcinoma study provides complementary evidence linking reduced CTSB activity with incomplete flux. However, the limited number of cancer-specific studies prevents generalization across tumor types.

These findings create an important therapeutic paradox. Inhibition of lysosomal CTSB may weaken nutrient recycling and tumor growth in cancers that depend on CTSB-supported autophagic flux. Conversely, pharmacological interference may produce treatment resistance when lysosomal sequestration or other adaptive responses dominate. Evaluation of CTSB targeting must therefore establish not only whether autophagic flux decreases but also how this change affects metabolism, drug distribution, and long-term cancer-cell survival.

5.4 Cathepsin B in Autophagy-Associated Apoptosis and Lysosomal Cell Death

Cathepsin B can participate in cancer-cell death through a mechanism that differs fundamentally from inhibition of lysosomal degradation. When cathepsin B remains confined within intact lysosomes, reduction of its activity may impair autophagic cargo degradation. In contrast, lysosomal membrane permeabilization can release active cathepsin B into the cytosol, where it gains access to death-regulatory proteins and may promote mitochondrial dysfunction, caspase activation, or degradation of oncogenic substrates. Therefore, the biological effect depends not only on CTSB activity but also on lysosomal membrane integrity and enzyme localization.

Autophagy-associated apoptosis was demonstrated directly in SPARC-overexpressing primitive neuroectodermal tumor cells. SPARC expression increased autophagic vacuoles, LC3 abundance, and cathepsin B expression before the development of mitochondrial and apoptotic changes. Inhibition of early autophagy with 3-methyladenine reduced both the autophagic response and subsequent apoptosis, indicating that activation of the autophagic machinery occurred upstream of cell death [12].

Cathepsin B inhibition produced a different result. Pharmacological suppression of CTSB reduced cytochrome-c release, caspase-3 activation, PARP cleavage, and apoptosis but did not prevent LC3 accumulation or autophagic-vacuole formation. Cathepsin B therefore appeared to function downstream of autophagy as a molecular connection to mitochondria-mediated apoptosis rather than as an essential component of autophagosome formation [12]. This distinction is central to the thesis: CTSB inhibition may suppress a death-promoting pathway even while the upstream autophagic response remains detectable.

The SPARC study nevertheless has methodological limitations. It relied partly on CA-074Me, whose intracellular selectivity is condition-dependent, and several autophagy conclusions were based on autophagosome-associated markers rather than a complete modern flux analysis [12,13,77]. Furthermore, SPARC overexpression represents a specific experimental context. The findings therefore provide strong evidence for CTSB acting downstream of autophagy in this model but do not establish that autophagy generally activates CTSB-dependent apoptosis in all cancers.

A related relationship between cysteine cathepsins, apoptosis, and autophagy was observed in camptothecin-treated MCF-7 breast cancer cells. Camptothecin rapidly increased cathepsin B

activity. Broad cysteine-cathepsin inhibition with E64d reduced BAX- and BID-associated mitochondrial events and decreased apoptosis while increasing autophagy-associated structures and LC3-related markers. BID knockdown similarly suppressed apoptosis and shifted the cellular response toward greater autophagic-marker accumulation [100]. These findings support a cathepsin–BID axis that influences whether treatment-stressed cells progress toward apoptosis or remain in an autophagy-dominant state. However, because E64d inhibits several cysteine cathepsins, this study does not prove that CTSB alone controlled the switch.

Cathepsin B-mediated death can also occur after lysosomal membrane permeabilization without evidence that autophagy is the initiating mechanism. In chronic myelogenous leukemia cells, pharmacological or genetic suppression of BCR–ABL induced lysosomal membrane permeabilization, CTSB activation, and redistribution of the enzyme into the cytosol. Cytosolic cathepsin B directly contributed to BCR–ABL degradation. CA-074Me treatment or CTSB knockdown partially protected K562 cells from imatinib-induced death, whereas CTSB overexpression increased treatment sensitivity [32]. Comparable CTSB activation and protection after inhibition were observed in primary CD34-positive cells from patients with chronic myelogenous leukemia.

This study provides strong evidence that cytosolic CTSB can become a treatment-execution mechanism. It combined pharmacological inhibition, genetic depletion, enzyme overexpression, detection of cytosolic redistribution, substrate cleavage, and primary patient-derived cells [32]. Nevertheless, it was not designed as an autophagic-flux study. It should therefore be interpreted as evidence for lysosome-dependent cell death rather than proof of autophagy-mediated apoptosis.

Siramesine provides an additional pharmacological example. Treatment of immortalized and malignant cells caused oxidative stress, lysosomal membrane permeabilization, and predominantly caspase-independent cell death. The CTSB-directed inhibitors CA-074Me and R-2525 produced partial but significant protection in MCF-7 cells, supporting a functional contribution of released cathepsin B [105]. Partial rather than complete protection indicated that oxidative damage and additional lysosomal enzymes or death pathways also contributed to the final outcome.

More recent evidence was obtained in acute lymphoblastic leukemia cells through alteration of the lysosomal two-pore channel TPC2. Loss of TPC2 increased lysosomal pH and volume, reduced lysosomal sequestration of weak-base chemotherapeutic drugs, and increased susceptibility to lysosomal damage. Following doxorubicin exposure, TPC2-deficient cells displayed increased cytosolic CTSB activity, BID truncation, caspase-3 activation, and PARP cleavage. CA-074Me partially reversed apoptosis, supporting a CTSB-dependent component of the sensitization mechanism [111].

The TPC2 study illustrates that lysosomal dysfunction can produce two simultaneous therapeutic effects: increased availability of a chemotherapeutic drug outside lysosomes and increased CTSB-mediated lysosomal cell death. However, TPC2 rather than CTSB was the initial target, and CA-074Me does not provide absolute enzyme specificity. The findings therefore support cytosolic CTSB as a downstream execution factor rather than demonstrating direct pharmacological targeting of CTSB.

Collectively, these studies reveal two mechanistically distinct CTSB-related outcomes. Reduced activity of lysosome-confined CTSB may impair terminal autophagic degradation, whereas release of active CTSB after lysosomal membrane permeabilization may promote cancer-cell death. The first mechanism is associated mainly with cargo accumulation and defective recycling; the second involves access to cytosolic substrates and activation of apoptotic or non-apoptotic death pathways.

This distinction has major therapeutic implications. Combining a CTSB inhibitor with a treatment that depends on cytosolic cathepsin B release could antagonize cancer-cell killing. Conversely, promoting controlled lysosomal membrane permeabilization may exploit the elevated lysosomal protease content of selected tumors. Studies must therefore establish whether CTSB remains inside intact lysosomes or has been released into the cytosol before inhibition is classified as therapeutically beneficial or harmful.

Finally, the coexistence of autophagy markers and apoptosis does not establish autophagy-dependent cell death. A causal relationship requires evidence that genetic or pharmacological suppression of essential autophagy machinery reduces the subsequent death response. When CTSB inhibition reduces apoptosis without altering upstream autophagy markers, the most accurate interpretation is that cathepsin B acts downstream of or parallel to the autophagic response.

5.5 Cancer-Type-Specific Patterns and Context Dependence

The experimental evidence reviewed in the preceding sections does not support a single universal outcome of cathepsin B modulation across cancer types. Reduced CTSB activity has been associated with impaired autophagic degradation, increased treatment sensitivity, metabolic disruption, drug resistance, or reduced apoptosis. These apparently conflicting outcomes become more understandable when the cancer model, pharmacological intervention, CTSB localization, autophagic function, and dominant cell-death mechanism are considered together.

In ovarian cancer models, the predominant reported outcome is late-stage autophagic blockade accompanied by increased treatment sensitivity. Bortezomib indirectly reduced CTSB expression through sustained ERK phosphorylation, impaired p62 clearance, and enhanced the anticancer effect of cisplatin [9]. Elaiophyllin similarly caused LC3-II and p62 accumulation, impaired cathepsin maturation and lysosomal function, and increased cell death under selected treatment conditions [97,98]. These studies support the therapeutic potential of disrupting lysosomal recycling in ovarian cancer. However, neither intervention selectively targeted CTSB, and both affected broader signaling or lysosomal systems. The evidence therefore supports a CTSB-associated lysosomal mechanism rather than exclusive dependence on cathepsin B.

Pancreatic ductal adenocarcinoma displays a different pattern. In this cancer type, preserved CTSB activity was shown to support productive autophagic flux, glycolytic metabolism, and tumor growth through CTSB-dependent regulation [11]. This finding is consistent with the high autophagy-lysosome dependency previously demonstrated in pancreatic cancer. In this context, CTSB inhibition could weaken metabolic recycling and suppress tumor growth rather than merely produce nonspecific lysosomal stress. Nevertheless, the principal intervention involved CTSB manipulation rather than direct pharmacological inhibition of CTSB, and the mechanism requires validation using selective lysosomal CTSB inhibitors.

In multidrug-resistant hepatocellular cancer cells, ADCX indirectly reduced CTSB through Akt overactivation and impaired autophagic degradation [10]. The resulting cargo accumulation was associated with increased apoptotic cell death. This study supports the possibility that resistant cancer cells depend on CTSB-containing lysosomal degradation systems. However, the evidence was derived primarily from HepG2/ADM cells, and the extent to which the mechanism applies to treatment-sensitive liver cancer or primary tumors remains uncertain.

Clear-cell renal-cell carcinoma illustrates that lysosomal dysfunction may contribute to treatment resistance rather than sensitization. Sunitinib accumulated within lysosomes, reduced lysosomal proteolytic function and CTSB activity, and was associated with incomplete autophagic flux [99]. Lysosomal sequestration simultaneously reduced the pharmacologically available drug concentration. Thus, inhibition of terminal autophagic degradation coexisted with a protective drug-storage mechanism. This finding demonstrates that reduced flux cannot automatically be interpreted as therapeutically beneficial; the effect of lysosomal drug distribution must also be considered.

In primitive neuroectodermal tumor cells, cathepsin B occupied a position downstream of the autophagic response. SPARC overexpression induced autophagy-associated changes followed by apoptosis, while CTSB inhibition reduced mitochondrial and caspase-dependent death without preventing the upstream autophagic response [12]. In this model, CTSB inhibition would be expected to protect malignant cells rather than enhance killing. The study therefore provides an important counterexample to the assumption that CTSB suppression is always therapeutically advantageous.

Hematological cancer models provide particularly strong evidence for death-promoting cytosolic CTSB. In chronic myelogenous leukemia, imatinib-induced lysosomal membrane permeabilization released cathepsin B into the cytosol, where it contributed to BCR–ABL cleavage and cancer-cell elimination [32]. In acute lymphoblastic leukemia, TPC2 loss increased chemotherapy-induced lysosomal damage, cytosolic CTSB activity, BID processing, and apoptosis [111]. These findings concern lysosome-dependent cell death rather than impairment of autophagic cargo degradation. Inhibiting CTSB during such responses could antagonize treatment efficacy.

Breast cancer experiments similarly indicate that CTSB-related outcomes depend on the treatment mechanism. In camptothecin-treated MCF-7 cells, broad cysteine-cathepsin inhibition reduced apoptotic signaling while increasing autophagy-associated structures [100]. Siramesine-induced lysosomal leakage and oxidative stress were also partially reduced by CTSB-directed inhibitors [105]. Because the inhibitors used were not fully selective and the treatments caused broader lysosomal injury, these studies support a contribution of CTSB-containing protease activity to cancer-cell death but do not establish an exclusively CTSB-dependent mechanism.

The observed differences should not be interpreted as fixed properties of individual cancer types. The studies vary in pharmacological compound, inhibitor selectivity, treatment duration, basal autophagic activity, genetic background, and experimental endpoint. For example, ovarian cancer studies mainly examined indirect lysosomal suppression, whereas hematological studies frequently examined lysosomal membrane permeabilization and cytosolic enzyme release. The opposite outcomes may therefore reflect different experimental mechanisms as much as intrinsic tissue-specific biology.

Across the available cancer models, five variables appear particularly important for predicting the outcome of CTSB modulation:

1. whether CTSB is inhibited directly or altered indirectly;
2. whether the affected enzyme is lysosomal, extracellular, or cytosolic;
3. whether autophagy is cytoprotective, cytotoxic, cytostatic, or functionally irrelevant;
4. whether other lysosomal proteases can compensate for reduced CTSB activity;
5. whether the anticancer treatment depends on lysosomal degradation or lysosomal membrane permeabilization.

The strongest causal evidence is obtained when pharmacological findings are supported by genetic manipulation, enzyme-activity measurements, dynamic autophagic-flux assays, and functional rescue. The CSTB–CTSB study in pancreatic cancer, the CTSB rescue experiments in bortezomib-treated ovarian cancer, and the genetic and pharmacological analyses in chronic myelogenous leukemia fulfil several of these criteria [9,11,32]. By contrast, studies relying primarily on CA-074Me, steady-state LC3-II accumulation, or total CTSB expression provide weaker target-specific evidence.

Overall, the current literature supports a context-dependent model rather than a universal therapeutic rule. Inhibition of lysosomal CTSB may be beneficial when cancer cells depend on CTSB-supported cargo degradation and metabolic recycling. The same intervention may be ineffective when other proteases compensate and potentially harmful when cytosolic CTSB participates in treatment-induced apoptosis. Cancer type alone is therefore insufficient for patient or model selection; the relevant CTSB pool, lysosomal phenotype, autophagic function, and treatment mechanism must be experimentally defined

5.6 Comparative Evaluation of the Original Evidence

The available original studies differ substantially in cancer model, targeting strategy, CTSB compartment, autophagy methodology, and final biological outcome. Direct comparison is therefore necessary to distinguish evidence that CTSB supports productive autophagic degradation from evidence that CTSB is merely one component of broader lysosomal dysfunction or acts downstream in lysosome-dependent cell death. Table 1 summarizes the principal studies according to intervention, mechanistic evidence, cellular outcome, and strength of CTSB-specific attribution.

Table 1. Comparative evaluation of original studies linking cathepsin B modulation to autophagy, lysosomal function, and cancer-cell fate

Study and experimental model	CTSB-related intervention	Autophagy and lysosomal evidence	Principal biological outcome	CTSB-specific evidence appraisal
Kao et al. [9]; ovarian cancer cells and tumor model	Bortezomib indirectly reduced CTSB expression through sustained ERK phosphorylation; CTSD was also reduced	LC3-II and p62 accumulation, impaired cargo degradation, lysosomal measurements, and partial restoration after CTSB overexpression	Increased cisplatin cytotoxicity and antitumor activity	Moderate-to-strong CTSB contribution. Rescue supports causality, but the intervention affected additional lysosomal enzymes and lacked a complete modern flux-assay panel
Sun et al. [10]; multidrug-resistant HepG2/ADM cells	ADCX indirectly reduced CTSB expression through Akt overactivation	Autophagosome and p62 accumulation with impaired autophagic degradation	Increased apoptotic death in resistant liver cancer cells	Moderate evidence. Cancer-specific but based mainly on one resistant cell model; direct CTSB binding and selective rescue were not demonstrated

Study and experimental model	CTSB-related intervention	Autophagy and lysosomal evidence	Principal biological outcome	CTSB-specific evidence appraisal
Jiang et al. [11]; pancreatic ductal adenocarcinoma cells, xenografts, and patient-derived models	CSTB sustained CTSB proteolytic activity by modifying its interaction with cystatin C	Dynamic flux assessment, lysosomal CTSB activity, bafilomycin experiments, cargo processing, and metabolic analysis	Increased glycolysis, proliferation, and tumor growth	Strongest evidence for CTSB-supported productive flux. Multiple functional assays were used, but CSTB rather than CTSB was the primary manipulated target
Bhoopathi et al. [12]; SPARC-overexpressing primitive neuroectodermal tumor cells	SPARC increased CTSB; CA-074Me or CTSB suppression reduced its function	Autophagic vacuoles and LC3-associated changes occurred upstream of CTSB-dependent apoptosis	Reduced CTSB activity protected cells from mitochondrial and caspase-dependent apoptosis	Moderate evidence for downstream CTSB function. The study supports an autophagy-to-apoptosis connection but used CA-074Me and limited dynamic-flux measurements
Lamore and Wondrak [93]; nonmalignant Hs27 fibroblasts	UVA exposure or CA-074Me reduced CTSB activity	LC3-II, p62, LAMP1, lysosomal expansion, and accumulation of undegraded substrates	Autophagic-lysosomal dysregulation	Mechanistic supporting evidence only. The model was nonmalignant, and CA-074Me may inhibit additional cysteine cathepsins
Lao et al. and Han et al. [94,95]; HeLa and other cancer models	Oblongifolin C impaired lysosomal function and was later shown to interact with purified CTSB	LC3-II and p62 accumulation, impaired fusion, altered lysosomal acidification, and reduced proteolysis	Increased apoptosis and sensitization to nutrient deprivation; antitumor activity in vivo	Strong evidence for late-stage blockade but limited CTSB specificity. The compound affected several lysosomal and nonlysosomal targets, and CTSB rescue was not demonstrated
Yu et al. [96]; cisplatin-sensitive and resistant esophageal cancer cells	Obatoclox accumulated in lysosomes and reduced cathepsins B, D, and L	Lysosomal clustering and simultaneous LC3-II and p62 accumulation; individual cathepsin knockdown affected LC3-II	Reduced viability and blockade of lysosomal autophagy	Strong evidence for broad lysosomal dysfunction but weak CTSB-specific attribution. Effects persisted in cells lacking detectable CTSB
Zhao et al. and Li et al. [97,98]; ovarian cancer cells and tumor models	Elaiophyllin indirectly impaired CTSB and CTSD maturation or activity through broader lysosomal and SHP2-MAPK-associated stress	LC3-II and p62 accumulation, impaired lysosomal proteolysis, membrane abnormalities, and late-stage flux blockade	Apoptosis or paraptosis and increased antitumor activity	Strong evidence for late-stage autophagy blockade. CTSB impairment occurred downstream and was not established as the exclusive initiating mechanism
Giuliano et al. [99]; clear-cell renal-cell carcinoma	Lysosomal sunitinib accumulation was associated with reduced CTSB activity and lysosomal dysfunction	Incomplete autophagic flux and lysosomal drug sequestration	Development of sunitinib resistance	Moderate CTSB-associated evidence. Reduced CTSB activity coincided with broader lysosomal changes and altered intracellular drug distribution
Puissant et al. [32]; chronic myelogenous leukemia cells and patient-derived cells	Imatinib induced lysosomal membrane permeabilization and cytosolic CTSB release	The study focused on lysosomal permeabilization rather than complete autophagic-flux analysis	Cytosolic CTSB cleaved BCR-ABL and promoted leukemia-cell death	Strong evidence for death-promoting cytosolic CTSB. Highly relevant to localization but not direct evidence concerning terminal autophagic degradation
Geisslinger et al. [111]; acute lymphoblastic leukemia cells	TPC2 loss enhanced lysosomal damage and cytosolic CTSB activity after chemotherapy	Lysosomal dysfunction was assessed, but autophagic flux was not the principal endpoint	Increased BID processing, caspase activation, and chemotherapy-induced apoptosis	Moderate evidence for downstream CTSB involvement. TPC2 was the initial target, and protection by CA-074Me was only partial

Evidence-appraisal criteria: Strong evidence indicates confirmation of CTSB activity or localization together with genetic manipulation, rescue, or convergent pharmacological evidence and functional outcome assessment. Moderate evidence indicates a plausible CTSB contribution supported by several experimental findings but lacking complete target-specific validation. Limited evidence indicates indirect CTSB alteration, broad lysosomal dysfunction, nonselective pharmacology, or absence of direct causal validation. The rating refers to CTSB-specific causal attribution and not to the overall quality of the study.

Abbreviations: ADCX, 25-O-acetyl-7,8-didehydrocimigenol-3-O-β-D-xylopyranoside; CSTB, cystatin B; CTSB, cathepsin B; CTSD, cathepsin D; CTSL, cathepsin L; ERK, extracellular signal-regulated kinase; LC3, microtubule-associated protein 1 light chain 3; LMP, lysosomal membrane permeabilization; PNET, primitive neuroectodermal tumor; TPC2, two-pore channel 2.

The comparison reveals that most cancer studies do not examine selective pharmacological inhibition of lysosomal CTSB in isolation. Bortezomib, ADCX, obatoclox, elaiophyllin, sunitinib, and oblongifolin C alter signaling pathways or several aspects of lysosomal function simultaneously. Their results provide biologically relevant evidence that CTSB impairment can accompany reduced autophagic degradation, but they do not consistently establish CTSB as the sole initiating target [9,10,94–99].

The study by Jiang et al. provides the most comprehensive evidence that preserved CTSB activity can support productive autophagic flux. It combined CTSB activity measurements with dynamic autophagy experiments, metabolic analyses, genetic manipulation, and tumor models. However, the principal experimental target was CSTB, and direct pharmacological inhibition of lysosomal CTSB was not tested with a highly selective inhibitor [11].

Kao et al. provide comparatively strong evidence that CTSB reduction contributes to late-stage autophagic impairment because restoration of CTSB partially improved cargo degradation and cancer-cell survival. Nevertheless, bortezomib also altered additional lysosomal enzymes, preventing exclusive attribution of the phenotype to CTSB [9].

The PNET, chronic myelogenous leukemia, and acute lymphoblastic leukemia studies demonstrate that CTSB can instead promote cancer-cell death after an autophagic response or lysosomal membrane permeabilization [12,32,111]. These findings concern a cytosolic or downstream death-promoting CTSB pool rather than lysosome-confined CTSB responsible for terminal cargo degradation. They explain why CTSB inhibition may protect cells in some treatment settings despite impairing lysosomal recycling in others.

Methodological quality also varies considerably. Some studies used dynamic flux assays, lysosomal-function measurements, genetic manipulation, or functional rescue, whereas others relied primarily on LC3-II, p62, autophagic vacuoles, or CA-074Me. Because CA-074Me does not maintain complete CTSB selectivity under all cellular conditions, findings based mainly on this inhibitor require support from genetic or activity-based approaches [12,13,77,93]. The nonmalignant fibroblast study provides useful mechanistic support but cannot be treated as direct cancer evidence.

The collective evidence supports three principal patterns. First, lysosomal CTSB can contribute to productive cargo degradation and metabolic recycling, as demonstrated most clearly in pancreatic cancer. Second, indirect suppression of a CTSB-containing lysosomal system can contribute to late-stage autophagic blockade, particularly when several lysosomal functions are altered simultaneously. Third, cytosolic CTSB released after lysosomal damage can promote apoptosis or lysosome-dependent cell death.

A major unresolved gap remains. None of the reviewed cancer studies fully combines a highly selective lysosome-accessible CTSB inhibitor, direct confirmation of intracellular target engagement, measurement of related cathepsins, dynamic autophagic-flux assays, genetic rescue, and validation across several tumor models. This is a critical synthesis of the available evidence rather than proof that such an experiment is impossible. It explains why the literature supports a context-dependent mechanistic relationship but does not yet establish a universal therapeutic effect of selective lysosomal CTSB inhibition.

Accordingly, the available studies should not be summarized by stating simply that CTSB inhibition either induces or suppresses autophagy. A more accurate conclusion is that the

outcome depends on the affected CTSB pool, degree of lysosomal protease redundancy, mechanism of pharmacological intervention, functional role of autophagy, and dependence of the treatment response on lysosomal membrane permeabilization.

6. Mechanistic Synthesis of Cathepsin B–Autophagy Interactions

6.1 Productive Autophagic Flux versus Autophagosome Accumulation

The central mechanistic problem in interpreting cathepsin B-related autophagy studies is the distinction between increased pathway throughput and accumulation caused by defective clearance. Productive autophagic flux requires coordinated autophagosome formation, fusion with lysosomes, degradation of the enclosed cargo, and recycling of the resulting metabolites. An increase in autophagosomes represents only the abundance of an intermediate compartment and cannot independently demonstrate that the complete pathway has become more active [1].

Lysosomal cathepsin B is positioned principally at the terminal degradative stage rather than within the core machinery responsible for autophagy initiation. Reduction of its activity may therefore leave ULK1 activation, phagophore expansion, LC3 lipidation, and autophagosome formation intact while decreasing the efficiency of cargo degradation after lysosomal delivery. Under these conditions, early autophagic input continues, but terminal processing becomes slower than the rate at which new cargo enters the pathway.

This imbalance produces a characteristic accumulation phenotype. LC3-II may increase because autophagosomal membranes are generated but not efficiently degraded, while p62 and other cargo substrates accumulate because lysosomal clearance is incomplete. Autophagosomes or autolysosomes may also become enlarged or more numerous. Such findings are compatible with late-stage blockade, but they become convincing only when supported by dynamic flux experiments and direct evidence of impaired lysosomal degradation [1,54,57].

Several cancer studies fit this mechanistic pattern. Bortezomib-treated ovarian cancer cells displayed reduced CTSB expression together with LC3-II and p62 accumulation, and restoration of CTSB partially improved cargo clearance [9]. ADCX similarly reduced CTSB expression and impaired autophagic degradation in multidrug-resistant liver cancer cells [10]. Oblongifolin C, obatoclast, and elaiophyllin produced comparable accumulation patterns together with defects in lysosomal acidification, protease activity, maturation, fusion, or membrane integrity [94,96–98].

These compounds nevertheless illustrate why marker accumulation cannot be attributed automatically to selective CTSB inhibition. Each intervention affected additional signaling pathways, lysosomal enzymes, or structural components of the autophagy–lysosome system. The resulting phenotype may therefore reflect combined reduction of cathepsin activity, altered lysosomal pH, defective fusion, membrane damage, or general loss of degradative capacity. CTSB impairment may contribute substantially without representing the only rate-limiting defect.

The opposite pattern is productive flux supported by lysosomal CTSB activity. In pancreatic ductal adenocarcinoma, CSTB-mediated preservation of CTSB activity increased autophagic cargo degradation and supported glycolytic metabolism and tumor-cell growth [11]. In this model, the relevant outcome was not greater accumulation of autophagic structures but more efficient passage of material through the pathway. Disruption of terminal lysosomal processing removed the proliferative advantage associated with CSTB overexpression.

These two patterns can be expressed as a balance between autophagic input and lysosomal output. When cargo delivery and degradation increase together, productive flux rises. When cargo delivery continues but lysosomal processing decreases, autophagic intermediates accumulate. When both formation and degradation decline, LC3-II abundance may remain unchanged or even decrease despite substantial suppression of the pathway. Steady-state marker levels therefore cannot reveal the direction of flux without comparison under conditions in which lysosomal turnover is experimentally blocked.

The relationship may also change over time. A pharmacological treatment can initially activate autophagosome formation as an adaptive stress response and later impair lysosomal degradation through progressive enzyme suppression, loss of acidity, or membrane damage. Measurements obtained at an early time point may consequently indicate increased flux, whereas later measurements show cargo accumulation and pathway failure. Dose and exposure duration must therefore be incorporated into the mechanistic interpretation.

Cellular outcome represents a separate analytical level. Productive flux may support survival by providing metabolites, but it may also participate in cytostatic or death-associated responses. Similarly, late-stage blockade may increase treatment sensitivity through metabolic deprivation and accumulation of damaged material, or it may promote resistance through lysosomal drug sequestration and adaptive signaling, as observed with sunitinib [99]. The direction of autophagic flux alone is therefore insufficient to predict therapeutic benefit.

A rigorous mechanistic interpretation should evaluate three related but distinct variables:

1. **Autophagic input:** whether autophagosome formation and cargo sequestration increase or decrease.
2. **Lysosomal processing:** whether fusion, acidification, cathepsin activity, cargo proteolysis, and metabolite recycling remain functional.
3. **Biological output:** whether the resulting pathway state promotes survival, growth arrest, apoptosis, lysosome-dependent death, or treatment resistance.

Applying this framework prevents the terms “autophagy induction” and “autophagy inhibition” from being used as oversimplified descriptions. Following cathepsin B modulation, increased LC3-II or autophagosome abundance should be interpreted first as accumulation of pathway intermediates. Only dynamic evidence demonstrating increased lysosomal turnover justifies classification as productive autophagic flux.

6.2 Lysosomal Dysfunction and Cargo-Clearance Failure

Failure of autophagic cargo clearance can arise at several mechanistically distinct stages. Autophagosomes may fail to fuse with lysosomes, lysosomal acidification may become insufficient, hydrolase maturation or catalytic activity may decline, or lysosomal membranes may lose their integrity. Each defect can produce accumulation of LC3-II, p62, and autophagic compartments, but the molecular meaning of this accumulation differs according to the affected stage.

Cathepsin B is most directly related to the proteolytic phase that follows autophagosome–lysosome fusion. Therefore, reduced CTSB activity can theoretically permit formation of an autolysosome while limiting degradation of the internal membrane and enclosed cargo. This

represents a post-fusion degradative defect rather than a failure of autophagosome formation or membrane fusion.

The bortezomib study provides the clearest cancer-specific example of this distinction. In ovarian cancer cells, bortezomib did not prevent autophagosome–lysosome fusion but reduced lysosomal cathepsin levels and impaired p62 degradation. Restoration of CTSB partially improved cargo clearance, supporting the conclusion that insufficient lysosomal proteolysis contributed to the blocked flux [9]. However, the simultaneous reduction of other lysosomal enzymes indicates that CTSB was one component of a broader degradative defect.

A related post-fusion defect was reported in multidrug-resistant HepG2/ADM cells. ADCX reduced CTSB expression through Akt overactivation and caused accumulation of autophagic cargo [10]. This finding supports the principle that signaling-mediated suppression of CTSB can reduce terminal degradation. Nevertheless, because direct restoration of CTSB activity and comprehensive analysis of additional lysosomal enzymes were limited, the exact proportion of the phenotype attributable specifically to CTSB remains uncertain.

Mechanistic studies of cathepsin redundancy show why selective reduction of CTSB may produce only partial clearance failure. Combined inhibition or loss of cathepsins B and L caused enlarged lysosomal compartments, accumulation of LC3-II and lysosomal proteins, reduced degradation of internalized substrates, and impaired intracellular lipid trafficking [42]. Although this was not a cancer study, it demonstrates that broad disruption of the cysteine-cathepsin network produces a more extensive lysosomal phenotype than loss of one protease alone.

In some pharmacological models, proteolytic failure is accompanied by defective lysosomal acidification or membrane fusion. Oblongifolin C reduced lysosomal acidity and proteolytic capacity and interfered with autophagosome–lysosome fusion [94]. The resulting LC3-II and p62 accumulation therefore cannot be explained by CTSB inhibition alone. Instead, CTSB impairment occurred within a compound-induced failure affecting several stages of lysosomal maturation and degradation.

Obatoclox similarly produced a multi-component lysosomal defect. It accumulated within lysosomes, caused lysosomal clustering, reduced cathepsins B, D, and L, and induced accumulation of LC3-II, p62, polyubiquitinated proteins, and undegraded cellular material [96]. Because the effect persisted in cells with little or no detectable CTSB, the study shows that severe cargo-clearance failure can occur through disruption of the collective lysosomal protease system even when CTSB is not the dominant enzyme.

Elaiophyllin produced an additional phenotype in which reduced degradative activity was accompanied by lysosomal destabilization. The compound attenuated cathepsin activity, prevented effective p62 clearance, and promoted release of CTSB and CTSD from lysosomes into the cytosol [97]. Later experiments linked these effects to broader SHP2–MAPK-associated cellular stress [98]. In this situation, impaired cargo degradation and lysosomal membrane permeabilization occur together, creating a transition from lysosomal recycling failure toward lysosome-dependent cell death.

Cargo-clearance failure does not invariably sensitize cancer cells to treatment. In clear-cell renal-cell carcinoma, sunitinib accumulation within lysosomes was associated with incomplete autophagic flux and reduced CTSB activity, but lysosomal sequestration also

lowered the concentration of drug available to act on cytosolic targets [99]. Lysosomal dysfunction therefore contributed to resistance rather than straightforward therapeutic sensitization.

The cellular consequences of impaired clearance may include accumulation of p62, ubiquitinated proteins, damaged organelles, and incompletely degraded macromolecules. At the same time, reduced lysosomal breakdown may limit the release of amino acids and other metabolites required for biosynthesis and energy production. The relative importance of toxic cargo accumulation and metabolic deprivation depends on the basal lysosomal demand of the cancer cell and the duration of pathway inhibition.

The extent of the phenotype is also influenced by residual proteolytic capacity. If cathepsins L, D, and other hydrolases remain active, selective reduction of CTSB may slow the degradation of particular substrates without producing complete lysosomal failure. Conversely, simultaneous impairment of several cathepsins, acidification, and membrane stability is more likely to produce extensive accumulation of undegraded material. A severe autophagy phenotype produced by a compound affecting several lysosomal processes should therefore not be presented as proof of a nonredundant CTSB function.

Cargo-clearance failure should be evaluated through complementary measurements. These include confirmation of autophagosome–lysosome fusion, assessment of lysosomal pH and membrane integrity, determination of mature CTSB and related cathepsin activities, and measurement of degradation using p62, long-lived proteins, or other cargo substrates. Dynamic LC3 turnover remains necessary to establish whether pathway input continues while terminal output declines.

The available evidence supports two levels of CTSB attribution. In the first, CTSB reduction contributes directly to an identifiable post-fusion proteolytic defect, as supported most clearly by the partial rescue observed in bortezomib-treated ovarian cancer cells. In the second, reduced CTSB activity is one feature of broader lysosomal dysfunction involving several enzymes, acidification, fusion, or membrane stability. Distinguishing these levels prevents general lysosomal failure from being misclassified as selective pharmacological inhibition of cathepsin B.

Finally, persistent lysosomal dysfunction may alter the role of CTSB itself. While enzyme loss inside intact lysosomes reduces cargo degradation, membrane permeabilization can redistribute residual active CTSB into the cytosol and convert it into a mediator of cell-death signaling. This transition is examined in the following section.

6.3 Lysosomal Membrane Permeabilization and Cytosolic Cathepsin B

Lysosomal membrane permeabilization represents a mechanistic transition between lysosomal degradative dysfunction and lysosome-dependent cell death. While cathepsin B retained inside intact lysosomes contributes principally to controlled substrate degradation, membrane permeabilization redistributes the enzyme into the cytosol, where it gains access to proteins that are normally separated from lysosomal hydrolases. The biological consequence of CTSB activity therefore changes fundamentally when lysosomal compartmentalization is lost.

The effect of lysosomal membrane permeabilization depends on its extent, duration, and cellular context. Limited leakage may release a fraction of the lysosomal protease pool and permit selective cleavage of cytosolic substrates. More extensive membrane damage can cause broader release of cathepsins and other hydrolases, loss of lysosomal acidity, defective cargo degradation, and irreversible cellular injury. Cathepsin B retains measurable catalytic activity across acidic and near-neutral conditions, although its substrate preferences and catalytic efficiency change with pH [23]. It may therefore remain functionally active after redistribution from the lysosomal lumen.

Lysosomal membrane permeabilization may be induced by reactive oxygen species, lipid accumulation, lysosomotropic compounds, disruption of membrane proteins, or excessive stress within autolysosomes. Autophagy can oppose this process by removing damaged lysosomes through lysophagy, but under selected conditions it can instead contribute to lysosomal overload or transport membrane-destabilizing lipids into autolysosomes. Autophagy and lysosomal damage should therefore not be viewed as obligatorily antagonistic processes.

Direct evidence for autophagy-dependent autolysosomal destabilization was obtained in glioma cells treated with Δ^9 -tetrahydrocannabinol. Hernández-Tiedra et al. showed that the treatment altered sphingolipid metabolism and transferred a dihydroceramide-enriched membrane composition into autophagosomes and autolysosomes. This change promoted lysosomal membrane permeabilization, cytosolic cysteine-cathepsin activity, and subsequent apoptotic cell death. Inhibition of autophagy or sphingolipid synthesis reduced lysosomal permeabilization and death, whereas nutrient deprivation induced autophagy without producing the same destabilizing lipid phenotype [112]. The study therefore demonstrated that autophagy-associated cell death depended on the nature of the cargo and membrane composition rather than on autophagy activation alone.

A related mechanism was demonstrated with B10, a glycosylated derivative of betulinic acid, in U87MG glioma cells. B10 simultaneously induced autophagosome formation and impaired autophagic flux while destabilizing lysosomes and releasing cathepsins B and Z into the cytosol. CA-074Me reduced cell death, BID-associated changes, mitochondrial depolarization, and caspase activation. Moreover, depletion of ATG5, ATG7, or BECN1 reduced lysosomal permeabilization and death, indicating that the autophagic machinery contributed functionally to the lysosomal injury [114].

The B10 findings should nevertheless not be interpreted as proof of an exclusively CTSB-dependent mechanism. CA-074Me may inhibit additional cysteine cathepsins, and B10 caused release of more than one lysosomal protease. The partial protection provided by cathepsin inhibition indicates that cytosolic proteases contributed to the death response but acted together with mitochondrial, caspase-dependent, and non-apoptotic mechanisms.

More recent evidence was obtained in glioblastoma cells treated with pimozide or loperamide. Both compounds increased autophagic activity, caused lysosomal accumulation of cholesterol and bioactive sphingolipids, induced lysosomal membrane permeabilization, and released CTSB into the cytosol. Genetic deletion of ATG5 or ATG7 reduced both permeabilization and cell death, while cathepsin inhibitors also provided protection. In contrast, depletion of the lysophagy regulator VCP increased lysosomal damage and death, supporting a protective role for the removal of injured lysosomes [108].

These glioma studies establish an important sequence: treatment-induced autophagy or autolysosomal lipid accumulation can promote membrane destabilization; lysosomal proteases are subsequently released; and cytosolic cathepsin activity contributes to the execution or amplification of cancer-cell death. This sequence differs from late-stage blockade caused solely by insufficient intralysosomal proteolysis. In both situations, cargo clearance may decline, but only lysosomal membrane permeabilization redistributes CTSB toward nonlysosomal substrates.

Cancer-specific studies reviewed previously support several possible cytosolic CTSB functions. In chronic myelogenous leukemia, released CTSB contributed directly to BCR–ABL degradation after imatinib-induced lysosomal membrane permeabilization [32]. In acute lymphoblastic leukemia, enhanced cytosolic CTSB activity was associated with BID truncation, mitochondrial signaling, and chemotherapy-induced apoptosis after TPC2 depletion [111]. In SPARC-overexpressing primitive neuroectodermal tumor cells, CTSB acted downstream of the autophagic response and promoted mitochondrial and caspase-dependent apoptosis [12]. These findings demonstrate that CTSB inhibition may interrupt therapeutically useful death signaling in selected treatment contexts.

Cytosolic detection of cathepsin B alone is not sufficient to establish a causal role. Lysosomal membrane permeabilization commonly releases several proteases simultaneously, and CTSB-directed inhibitors may not remain completely selective inside living cells. Strong attribution requires demonstration of enzyme redistribution, measurement of cytosolic catalytic activity, comparison with related cathepsins, and functional testing using genetic depletion, rescue, or more selective chemical tools.

Methodologically, loss of LysoTracker staining should not be treated as definitive evidence of membrane permeabilization because reduced fluorescence may also result from lysosomal deacidification. More direct evidence includes cytosolic redistribution of CTSB by immunofluorescence, biochemical fractionation, increased cytosolic cathepsin activity, or recruitment of cytosolic galectins to damaged lysosomal membranes. Measurements should be performed at several time points to determine whether lysosomal leakage precedes mitochondrial dysfunction and cell death or occurs only as a secondary consequence.

The distinction between lysosomal CTSB inhibition and cytosolic CTSB release has direct therapeutic importance. Inhibiting lysosome-confined CTSB may reduce metabolic recycling in autophagy-dependent tumors. In contrast, inhibiting CTSB after treatment-induced lysosomal permeabilization may protect malignant cells from apoptosis or lysosome-dependent death. The same CTSB inhibitor may therefore enhance one anticancer strategy while antagonizing another.

The available evidence supports a localization-dependent model. Cathepsin B inside intact lysosomes primarily participates in controlled proteolysis, whereas cathepsin B released into the cytosol can become a mediator of stress signaling and cell death. Experimental studies must establish lysosomal membrane integrity and enzyme localization before a change in cancer-cell survival is attributed simply to increased or decreased CTSB activity.

6.4 Protease Redundancy and Compensatory Adaptation

Cathepsin B operates within a lysosomal protease network in which several enzymes possess partly overlapping degradative functions. Reduction of CTSB activity therefore does not

produce a fixed phenotype. The result depends on whether other proteases can process the relevant substrates, whether their catalytic capacity is sufficient for the amount of cargo entering lysosomes, and whether the cells have time to develop adaptive responses.

Protease redundancy and compensatory adaptation should be distinguished conceptually. Redundancy refers to pre-existing functional overlap that permits another enzyme to degrade a substrate immediately after CTSB activity is reduced. Adaptation refers to later cellular changes, such as increased expression, maturation, activity, or redistribution of another protease. Both mechanisms can preserve lysosomal degradation, but they differ in timing and experimental interpretation.

Evidence for compensatory protease activity was obtained in pancreatic ductal adenocarcinoma. Constitutive CTSB deficiency delayed tumor development and prolonged survival but did not completely prevent pancreatic cancer progression. CTSB-deficient tumors also displayed increased levels of active cathepsin L, suggesting that CTSL could preserve part of the proteolytic capacity lost after CTSB deletion [38]. This compensation may explain why the phenotype remained substantial but incomplete.

The effect of simultaneous cathepsin disruption supports this interpretation. Combined inhibition or loss of cathepsins B and L produced more extensive lysosomal abnormalities than disruption of either enzyme alone. These abnormalities included impaired degradation of internalized substrates, LC3-II and lysosomal-protein accumulation, enlarged acidic compartments, and altered cholesterol trafficking [42]. The severe phenotype indicates that the lysosomal system possesses a proteolytic reserve that can tolerate reduction of one enzyme more readily than combined loss of several related proteases.

In vivo genetic evidence further demonstrates the importance of this collective reserve. Mice lacking either cathepsin B or cathepsin L individually remained considerably less affected than animals deficient in both enzymes. Combined deficiency caused severe neurodegeneration, accumulation of autophagic-lysosomal material, growth impairment, and early mortality, while expression of human cathepsin L rescued the lethal phenotype [88]. These findings demonstrate functional overlap but also show that compensation becomes insufficient when the remaining proteolytic capacity falls below a critical level.

Compensation is not limited to the degradation of endogenous cellular cargo. Valine–citrulline linkers were historically described as cathepsin B-cleavable, yet removal of CTSB did not abolish intracellular processing or cytotoxicity of several antibody–drug conjugates. Other lysosomal cysteine proteases were able to release the payload in CTSB-deficient cells [84]. Although this experiment did not examine autophagy, it demonstrates that cleavage by purified CTSB does not establish exclusive enzyme dependence within the lysosomal environment.

The pharmacological studies reviewed in Chapter 5 provide additional evidence that severe autophagic blockade commonly reflects disruption of several lysosomal enzymes. Bortezomib reduced both CTSB and CTSD, while obatoclox decreased active cathepsins B, D, and L [9,96]. Obatoclox continued to impair autophagy in cancer cells with little or no detectable CTSB, demonstrating that other lysosomal defects could reproduce the accumulation phenotype independently of cathepsin B.

These findings explain why broad cysteine-cathepsin inhibitors may generate stronger autophagic phenotypes than selective CTSB inhibition. A compound that simultaneously suppresses cathepsins B and L may markedly reduce collective proteolysis and cause extensive LC3-II, p62, and cargo accumulation. The severity of this phenotype does not demonstrate that CTSB possesses an individually nonredundant function; it may instead reflect loss of several overlapping enzymes.

Conversely, a weak phenotype after selective CTSB inhibition does not establish that the enzyme is biologically irrelevant. Residual lysosomal proteases may maintain overall protein turnover while specific CTSB-dependent substrates or processes remain affected. Measurements of bulk cargo degradation may therefore conceal selective defects that become apparent only under high autophagic demand, metabolic stress, or combination treatment.

The duration of CTSB alteration is an important determinant of compensation. Acute pharmacological inhibition reduces catalytic activity before major transcriptional or proteomic adaptation is expected to occur, although pre-existing protease redundancy remains active. Chronic knockdown, gene deletion, or prolonged drug exposure allows cells to alter lysosomal-enzyme abundance and select populations capable of surviving with reduced CTSB function. Acute and chronic experiments may consequently produce different degrees of autophagic impairment even when CTSB activity is reduced to a similar extent.

Rescue experiments help distinguish compensation from direct CTSB dependence. Restoration of CTSB partially improved cargo degradation in bortezomib-treated ovarian cancer cells [9], supporting a genuine contribution of the enzyme. However, incomplete rescue is compatible with simultaneous impairment of other lysosomal components. Similarly, restoration of CTSB activity should not be considered conclusive when supraphysiological overexpression broadly increases lysosomal proteolysis or alters enzyme localization.

A rigorous assessment of compensation should therefore include measurement of the mature and active forms of cathepsins B, L, D, and other relevant hydrolases. These measurements should be performed at several time points after pharmacological or genetic intervention. Comparison of selective CTSB inhibition, combined cathepsin inhibition, transient depletion, chronic deletion, and CTSB rescue can reveal whether the observed phenotype depends on one enzyme or on the collective proteolytic capacity of the lysosome.

Protease compensation has direct therapeutic implications. Tumors with substantial lysosomal reserve may resist selective CTSB inhibition by maintaining degradation through alternative cathepsins. Broader inhibition could overcome this resistance but would also increase the risk of disrupting essential lysosomal functions in normal tissues. An effective strategy may therefore require identification of tumors in which CTSB performs a nonredundant function or combination treatment that reduces compensatory capacity without causing unacceptable systemic toxicity.

Overall, the available evidence supports CTSB as an important but context-dependent component of lysosomal proteolysis. Neither the absence nor the presence of an autophagy phenotype after CTSB targeting can be interpreted without evaluating the activity of other lysosomal proteases. Protease redundancy and adaptive compensation are therefore central explanations for differences between inhibitors, genetic models, cancer types, and treatment durations.

6.5 Crosstalk with Apoptosis, Ferroptosis, and Cellular Stress Responses

Cathepsin B–autophagy interactions occur within a broader network of cellular stress and regulated cell-death pathways. Oxidative stress, endoplasmic-reticulum stress, mitochondrial injury, lysosomal dysfunction, and altered lipid or iron metabolism can simultaneously modify autophagic flux and CTSB localization. Consequently, autophagy, apoptosis, and ferroptosis should not be interpreted as completely separate outcomes when several of these processes develop after the same treatment.

Autophagy frequently represents an early response to cellular stress. Moderate activation may remove damaged proteins and organelles and preserve cancer-cell survival. If the stress exceeds lysosomal processing capacity or destabilizes lysosomal membranes, the same autophagic response may instead contribute to cargo accumulation, lysosomal membrane permeabilization, and the release of cathepsins. The functional outcome therefore depends on whether autophagy successfully resolves the stress or contributes to lysosomal overload and cell-death signaling.

A principal connection between cytosolic cathepsin B and apoptosis involves the mitochondrial pathway. After lysosomal membrane permeabilization, CTSB and related proteases may promote BID cleavage, mitochondrial outer-membrane permeabilization, cytochrome-c release, and activation of caspases. This sequence was supported in SPARC-overexpressing primitive neuroectodermal tumor cells, camptothecin-treated breast cancer cells, imatinib-treated chronic myelogenous leukemia cells, and chemotherapy-treated acute lymphoblastic leukemia cells [12,32,100,111]. The strength of CTSB-specific attribution differs among these studies because several relied on broad cathepsin inhibitors or CA-074Me, whereas the chronic myelogenous leukemia study also included genetic CTSB manipulation.

An informative cancer-specific example was provided by IMB-6G in pancreatic cancer cells. IMB-6G caused autophagosome accumulation while blocking terminal degradation through attenuation of lysosomal cathepsin activity. Treatment subsequently induced lysosomal membrane permeabilization and cytosolic release of CTSB and CTSD. ATG5 depletion reduced lysosomal damage and apoptosis, while cathepsin inhibitors also partially protected the cells. These experiments support an autophagosome–lysosome–cathepsin axis in which autophagic accumulation contributes to membrane permeabilization and released lysosomal proteases promote apoptosis [115].

The IMB-6G study also demonstrates why autophagy blockade and autophagy-dependent death are not mutually exclusive descriptions. The compound prevented successful completion of autophagic degradation, but formation of autophagosomes was still required for maximal lysosomal injury and apoptosis. Thus, the pathway was blocked at its terminal stage while earlier autophagic machinery contributed functionally to the death response. The term “autophagy-dependent apoptosis” in this setting refers to the requirement for autophagosome formation, not to productive lysosomal recycling.

Oxidative stress can further amplify the interaction between autophagy and CTSB. Reactive oxygen species may damage lysosomal membranes, alter the redox state of the catalytic cysteine, impair mitochondrial function, and increase the burden of damaged material entering autophagy. In nutritionally stressed cells, thioredoxin-reductase inhibition increased lysosomal membrane permeabilization and CTSB activity while impairing protective

autophagic processing through differential effects on cysteine cathepsins [24]. Although this was not a cancer model, it demonstrates that CTSB protein abundance and catalytic activity may respond differently to oxidative stress.

Endoplasmic-reticulum and kinase-associated stress pathways may indirectly produce a similar transition. Elaiophyllin activated an SHP2–SOS1–MAPK stress pathway, induced endoplasmic-reticulum stress and paraptotic vacuolization, and impaired CTSB- and CTSD-containing lysosomal activity [98]. In this model, reduced cathepsin function and autophagy blockade were downstream components of a broader pharmacological stress response rather than evidence that CTSB was the initial drug target.

Ferroptosis introduces an additional relationship between autophagy, lysosomes, iron metabolism, and cathepsin B. Ferroptosis is driven by iron-dependent lipid peroxidation and failure of antioxidant protection. Autophagy can increase ferroptotic sensitivity through ferritinophagy, in which ferritin is delivered for lysosomal degradation and stored iron is released into the metabolically available iron pool. Hou et al. demonstrated that suppression of autophagy or ferritin degradation reduced ferroptosis in fibroblast and cancer-cell models, establishing ferritin turnover as one mechanism connecting autophagy with iron-dependent death [109].

Cathepsin B may participate downstream in ferroptotic execution. Nagakannan et al. reported that several ferroptosis inducers caused lysosomal membrane permeabilization and cytoplasmic leakage of CTSB. Pharmacological inhibition and genetic deletion of CTSB protected membrane integrity and reduced mitochondrial and chromatin damage, including cleavage of histone H3. However, much of this evidence was obtained in fibroblasts and neuronal models rather than cancer-specific systems. The study therefore provides strong mechanistic evidence for CTSB as a ferroptotic mediator but limited direct evidence for its therapeutic role in tumors [107].

Cancer-specific supporting evidence was reported in Ewing sarcoma cells treated with carbonic-anhydrase II inhibitors. The compounds reduced AKT–FTH1 signaling and produced findings consistent with ferroptotic cell death. CTSB abundance increased during treatment, and CTSB knockdown reduced the cytotoxic effect of selected inhibitors. This result supports a functional CTSB contribution but does not establish that CTSB was the initial trigger or the exclusive executor of ferroptosis. The pharmacological target was carbonic anhydrase II, and the experiments did not demonstrate selective manipulation of lysosomal CTSB activity independently of the wider iron- and stress-response pathway [108].

The relationship between CTSB and ferroptosis is therefore different from its role in terminal autophagic degradation. In productive autophagy, lysosomal CTSB contributes to controlled cargo proteolysis. During ferritinophagy, lysosomal degradation may increase the available iron pool and promote lipid peroxidation. Following lysosomal membrane permeabilization, CTSB may then relocate to the cytosol and amplify cellular injury. These events can occur sequentially but should not be treated as one inseparable mechanism.

Autophagy may also protect against ferroptosis by removing damaged mitochondria, lipid droplets, or injured lysosomes. The biological direction depends on the selective cargo being degraded. Ferritinophagy may promote iron release, whereas lysophagy may remove permeabilized lysosomes and limit cytosolic cathepsin leakage. An increase in general LC3-II

therefore does not reveal whether the dominant autophagic process is protective or ferroptosis-promoting.

These interactions create an important pharmacological dilemma. CTSB inhibition may reduce lysosomal nutrient recycling and weaken an autophagy-dependent tumor. The same inhibition may also suppress BID-dependent apoptosis or CTSB-associated ferroptotic execution and thereby protect cancer cells from another treatment. Combining a CTSB inhibitor with chemotherapy, lysosomotropic compounds, oxidative-stress inducers, or ferroptosis-inducing agents must therefore be evaluated experimentally rather than assumed to be synergistic.

A rigorous analysis should determine the temporal sequence of events. Measurements should establish whether oxidative or endoplasmic-reticulum stress precedes autophagy activation, whether lysosomal degradation remains functional, whether membrane permeabilization occurs before mitochondrial injury, and whether CTSB is retained inside lysosomes or released into the cytosol. Apoptosis should be assessed through mitochondrial and caspase markers, while ferroptosis requires evidence of lipid peroxidation, iron dependence, and rescue by appropriate ferroptosis inhibitors rather than reliance on reduced viability alone.

Overall, cathepsin B functions as a context-dependent connection between lysosomal degradation and several cell-fate pathways. Within intact lysosomes, it may support metabolic adaptation. Following cargo-clearance failure or membrane permeabilization, it may contribute to apoptosis, ferroptosis, or other forms of lysosome-dependent death. The therapeutic effect of CTSB targeting consequently depends on which of these functions dominates under the specific treatment conditions.

6.6 Context-Dependent Determinants of the Biological Outcome

The biological outcome of cathepsin B modulation cannot be predicted solely from whether CTSB activity increases or decreases. The same apparent reduction in enzyme activity may impair metabolic recycling, sensitize cancer cells to treatment, promote drug resistance, or suppress lysosome-dependent cell death. These different outcomes reflect interactions between enzyme localization, pharmacological selectivity, lysosomal capacity, autophagic function, treatment mechanism, and tumor-cell context.

The first determinant is the **subcellular CTSB pool affected by the intervention**. Inhibition of lysosome-confined CTSB may reduce terminal cargo proteolysis and nutrient recycling, as supported by the relationship between CTSB activity, productive autophagic flux, and pancreatic cancer metabolism [11]. Inhibition of extracellular or cell-surface CTSB may instead reduce matrix degradation and invasion without substantially changing autophagy [30,31]. Conversely, inhibition of cytosolic CTSB released after lysosomal membrane permeabilization may protect cancer cells from apoptosis or lysosome-dependent death [12,32,111].

The second determinant is the **nature of the targeting strategy**. Direct catalytic inhibitors, indirect signaling modulators, genetic depletion, and CTSB-responsive delivery systems are not mechanistically equivalent. Bortezomib and ADCX reduced CTSB expression indirectly through ERK- and Akt-associated signaling [9,10]. Obatoclax and elaiophylin affected several lysosomal enzymes and broader lysosomal properties [96–98]. Cathepsin-responsive

prodrugs, by contrast, exploit enzymatic activity and should not be interpreted as CTSB inhibition [82–87].

A third determinant is **selectivity and intracellular target engagement**. CA-074 may remain poorly accessible to intracellular CTSB during short exposure, while CA-074Me can enter cells but may inhibit cathepsin L and additional cysteine proteases under some conditions [13,75–77]. A severe autophagy phenotype caused by a nominal CTSB inhibitor may therefore reflect broad reduction of lysosomal proteolysis, whereas absence of a phenotype may reflect insufficient intracellular delivery.

The fourth determinant is the **remaining lysosomal proteolytic reserve**. Cathepsins B, L, D, and other hydrolases possess partially overlapping functions. Compensation by cathepsin L was observed after CTSB loss in pancreatic cancer, while combined cathepsin disruption produced more severe lysosomal abnormalities than loss of one enzyme alone [38,42,88]. Tumors with substantial proteolytic reserve may therefore tolerate selective CTSB inhibition more readily than tumors in which CTSB performs a rate-limiting or nonredundant role.

The fifth determinant is the **basal dependency of the tumor on autophagy and lysosomal recycling**. Pancreatic cancers and several RAS- or BRAF-driven tumor models display high autophagy–lysosome activity and metabolic dependence on intracellular recycling [3–5,63,66]. In such models, reducing terminal lysosomal degradation may create metabolic vulnerability. In tumors with lower basal dependency, CTSB inhibition may produce only limited metabolic consequences.

A sixth determinant is the **functional role of autophagy under the investigated treatment conditions**. If autophagy is cytoprotective, impairment of lysosomal degradation may increase treatment sensitivity. If autophagy contributes to cytostatic or cytotoxic signaling, its interruption may reduce therapeutic efficacy. Autophagy may also be nonprotective, in which case changes in LC3-II or p62 do not substantially determine survival. Functional classification must therefore be established experimentally rather than inferred from marker accumulation.

The seventh determinant is **lysosomal membrane integrity**. Reduction of CTSB activity within intact lysosomes primarily affects controlled proteolysis. After lysosomal membrane permeabilization, residual active CTSB may enter the cytosol and participate in BID processing, mitochondrial damage, oncogenic-protein cleavage, apoptosis, ferroptosis, or other lysosome-dependent death mechanisms [32,102–108]. A treatment that relies on cathepsin release may therefore be antagonized by simultaneous CTSB inhibition.

The eighth determinant is **intracellular drug distribution**. Lysosomes may sequester weakly basic drugs and reduce their availability at cytosolic or nuclear targets. In clear-cell renal-cell carcinoma, lysosomal accumulation of sunitinib was associated with incomplete autophagic flux, reduced CTSB activity, and treatment resistance [99]. Consequently, impaired lysosomal degradation may coexist with a pharmacokinetic resistance mechanism rather than producing straightforward sensitization.

The ninth determinant is **dose and exposure duration**. Acute inhibition may reduce CTSB catalytic activity before substantial compensatory adaptation occurs. Prolonged treatment may alter cathepsin expression, lysosomal biogenesis, membrane stability, and cellular metabolism. A compound may initially induce a productive stress response but later cause

lysosomal dysfunction and cargo accumulation. Conclusions based on one concentration and one time point may therefore fail to capture the transition between adaptation and pathway collapse.

The tenth determinant is the **cellular source of CTSB within the tumor microenvironment**. Tumor cells, macrophages, and other stromal populations may contribute distinct CTSB pools [36,37]. Systemic inhibition can affect malignant and nonmalignant cells simultaneously, potentially modifying invasion, inflammation, antigen processing, and treatment response. Results obtained from isolated cancer-cell cultures may not reproduce the contribution of host-derived CTSB in vivo.

Finally, the apparent outcome is strongly influenced by **experimental methodology**. Studies based only on CTSB expression, LC3-II accumulation, p62 abundance, or CA-074Me treatment provide limited causal evidence. Stronger conclusions require measurement of catalytic activity and localization, assessment of related cathepsins, dynamic autophagic-flux assays, evaluation of lysosomal pH and membrane integrity, and functional testing of survival or death. Genetic depletion and rescue can further strengthen attribution when compensatory effects are considered

Determinant	Key mechanistic question	Possible biological implication	Representative evidence
CTSB localization	Is the affected enzyme lysosomal, extracellular, cell-surface-associated, or cytosolic?	Lysosomal inhibition may reduce cargo degradation; extracellular inhibition may reduce invasion; cytosolic inhibition may suppress death signaling	[11,12,30–32,111]
Type of intervention	Is CTSB inhibited directly, altered indirectly, genetically depleted, or exploited for payload release?	Different interventions may affect catalytic activity, protein abundance, trafficking, or several lysosomal processes simultaneously	[9,10,82–87,96–98]
Cellular access and selectivity	Does the compound reach lysosomes and selectively inhibit CTSB?	Incomplete access may produce false-negative findings; broad cathepsin inhibition may exaggerate CTSB attribution	[13,72,75–77]
Protease redundancy	Can other cathepsins preserve lysosomal proteolysis?	Compensation may weaken the effect of selective CTSB inhibition; broad inhibition may produce stronger toxicity	[38,42,84,88]
Basal lysosomal dependency	Does the tumor require autophagic recycling for metabolism and growth?	High-dependency tumors may be more sensitive to disruption of terminal degradation	[3–5,11,63,66]
Functional role of autophagy	Is autophagy cytoprotective, cytotoxic, cytostatic, or nonprotective?	Blocking flux may enhance or reduce treatment efficacy depending on the functional role	[3–5,63–65]
Lysosomal membrane integrity	Is CTSB retained in intact lysosomes or released into the cytosol?	Cytosolic CTSB may promote apoptosis, oncogenic-protein cleavage, or ferroptotic damage	[12,32,102–108]
Drug sequestration	Does lysosomal accumulation alter the available concentration of the anticancer drug?	Lysosomal dysfunction may coexist with or promote pharmacological resistance	[99]
Dose and duration	Is the intervention acute or prolonged?	Early adaptive flux may be followed by late-stage blockade, compensation, or membrane damage	[9,94,97,98]
Cellular source	Is CTSB derived from cancer cells, macrophages, or other stromal cells?	Systemic targeting may alter both tumor-cell biology and the microenvironment	[36,37,89–92]
Experimental evidence	Were target engagement, dynamic flux, lysosomal function, and cell fate measured?	Methodological limitations determine the strength of CTSB-specific causal attribution	[1,13,57,77]

Table 2. Principal determinants of the outcome following cathepsin B modulation

Note: The listed determinants are interdependent and should not be interpreted as independent predictors of therapeutic response. “Representative evidence” refers to studies illustrating the mechanistic principle and does not indicate that the finding has been validated across all cancer types.

Abbreviations: CTSB, cathepsin B; LMP, lysosomal membrane permeabilization

The interaction of these determinants explains why apparently conflicting experimental results can all be biologically plausible. CTSB inhibition is most likely to produce therapeutic benefit when the tumor depends on lysosomal CTSB-supported recycling, the inhibitor reaches the relevant compartment selectively, compensation is limited, and the accompanying anticancer treatment does not require cytosolic CTSB for cell killing.

In contrast, CTSB inhibition may be ineffective when other lysosomal proteases maintain degradation and may become harmful when treatment-induced lysosomal membrane permeabilization depends on CTSB-mediated death signaling. The appropriate therapeutic strategy must therefore be selected according to the dominant CTSB function in the specific tumor and treatment context.

This mechanistic framework supports a central conclusion of the thesis: cathepsin B is not universally tumor-promoting or universally death-promoting. Its function is defined by localization, lysosomal state, treatment mechanism, and cellular dependency. These factors must be integrated before pharmacological CTSB targeting can be evaluated as a rational anticancer strategy

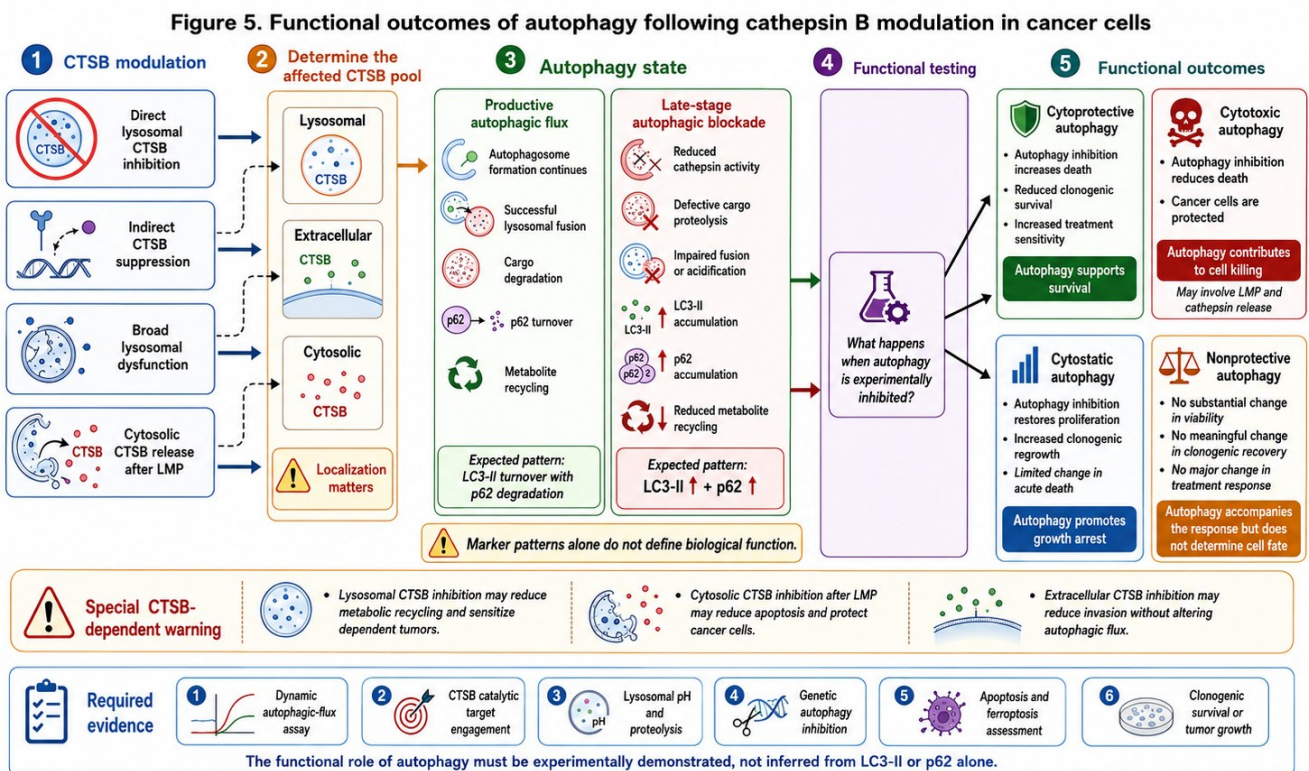


Figure 5. Functional outcomes of autophagy following cathepsin B modulation in cancer cells. Changes in cathepsin B activity may be associated with productive autophagic flux, late-stage autophagic blockade, lysosomal membrane permeabilization, or broader lysosomal dysfunction. The functional role of the resulting autophagic response cannot be determined from LC3-II, p62, or autophagosome abundance alone. Autophagy is classified as cytoprotective when its inhibition increases cancer-cell death or treatment sensitivity, cytotoxic when autophagy inhibition protects cells, cytostatic when it reverses prolonged growth arrest or clonogenic suppression, and nonprotective when pathway inhibition does not substantially alter cell fate. Interpretation must additionally consider whether the affected cathepsin B pool is lysosomal, extracellular, or cytosolic. Conceptual synthesis based on references [1,3–5,9–12,32,57,62–65,94–108]. Created by the author with the assistance of artificial intelligence and biorender.com

Figure 5. Functional outcomes of autophagy following cathepsin B modulation in cancer cells

7. Critical Discussion and Therapeutic Implications

7.1 Overall Strength and Limitations of the Evidence

The evidence connecting pharmacological targeting of cathepsin B with autophagy in cancer is biologically coherent but methodologically heterogeneous. Collectively, the available studies support a relationship between CTSB activity, lysosomal proteolysis, autophagic cargo clearance, metabolic adaptation, and cancer-cell fate. However, the evidence does not yet justify a universal conclusion that selective inhibition of lysosomal CTSB consistently blocks autophagic flux or improves anticancer efficacy.

The strongest mechanistic evidence is obtained when a study combines several complementary elements: measurement of catalytically active CTSB, confirmation of the affected intracellular compartment, dynamic assessment of autophagic flux, evaluation of lysosomal function, genetic manipulation or rescue, and measurement of the final cellular or tumor outcome. Only a limited number of the reviewed studies fulfil several of these criteria simultaneously.

The pancreatic ductal adenocarcinoma study by Jiang et al. provides the strongest evidence that preserved CTSB activity can support productive autophagic flux and cancer metabolism [11]. The study combined genetic manipulation, CTSB activity measurements, protein-interaction analysis, dynamic autophagy experiments, metabolic assessment, and tumor models. Nevertheless, CSTB rather than CTSB was the principal manipulated target. The findings therefore strongly support a CTSB-dependent biological pathway but do not directly establish the therapeutic effect of a selective lysosomal CTSB inhibitor.

The bortezomib study provides comparatively strong evidence for a CTSB contribution to impaired lysosomal cargo clearance [9]. CTSB expression decreased, p62 degradation was impaired, and CTSB restoration partially improved cargo processing and cancer-cell survival. The rescue experiment strengthens causal attribution. However, bortezomib also altered additional lysosomal enzymes and signaling pathways, preventing the autophagic phenotype from being attributed exclusively to CTSB.

Most other compounds discussed in Chapter 5 provide stronger evidence for general lysosomal dysfunction than for selective CTSB inhibition. Oblongifolin C, obatoclox, elaiophylin, ADCX, and sunitinib altered several processes, including signaling, cathepsin abundance or maturation, lysosomal acidification, membrane integrity, fusion, and intracellular drug distribution [10,94–99]. These studies are valuable for demonstrating that CTSB impairment can occur within treatment-induced lysosomal dysfunction, but they do not isolate CTSB as the sole initiating target.

A major pharmacological limitation is the reliance on CA-074Me. Although frequently described as a cell-permeable CTSB inhibitor, its intracellular selectivity varies according to concentration, exposure duration, cell type, redox conditions, and conversion to CA-074 [13,75–77]. Studies based primarily on protection by CA-074Me therefore demonstrate involvement of a CA-074Me-sensitive cysteine-protease system more confidently than they demonstrate an exclusive CTSB mechanism.

CA-074 presents the opposite limitation. It can retain greater CTSB selectivity under defined conditions but crosses cellular membranes inefficiently during short exposures. Failure of

CA-074 to alter an intracellular phenotype may consequently reflect insufficient lysosomal access rather than absence of CTSB involvement. Neither CA-074 nor CA-074Me should therefore be used as the only evidence for intracellular CTSB-specific causality.

Autophagy methodology represents another important weakness. Several studies measured LC3-II, p62, fluorescent puncta, or autophagic vacuoles without fully separating increased autophagosome formation from reduced lysosomal clearance. Dynamic flux experiments, matched treatment with late-stage inhibitors, tandem fluorescent reporters, and direct cargo-degradation assays were not applied consistently across the literature [1,57]. This limits comparison between studies and increases the risk that autophagosome accumulation is incorrectly described as autophagy induction.

Measurement of CTSB is similarly inconsistent. Total protein abundance does not necessarily reflect mature catalytic activity, and whole-cell enzyme assays do not identify whether the relevant activity is lysosomal, extracellular, or cytosolic. Several studies reported changes in CTSB expression without confirming intracellular target engagement or measuring related cathepsins. Such findings establish association more readily than causality.

Genetic evidence improves target specificity but introduces different limitations. Chronic CTSB knockdown or deletion can trigger adaptation by other lysosomal proteases, particularly cathepsin L [38]. Genetic loss may also affect CTSB protein localization and noncatalytic functions, whereas a pharmacological inhibitor primarily targets accessible catalytic activity. Agreement between pharmacological and genetic approaches is therefore especially valuable, but identical results should not automatically be expected.

Cancer-model heterogeneity further limits generalization. The reviewed studies include ovarian, pancreatic, hepatic, renal, esophageal, breast, glioma, leukemia, and primitive neuroectodermal tumor models. These cancers differ in basal autophagic activity, metabolic dependency, lysosomal abundance, oncogenic signaling, drug transport, and susceptibility to lysosomal membrane permeabilization. A mechanism established in pancreatic ductal adenocarcinoma cannot be transferred directly to a hematological cancer in which cytosolic CTSB contributes to treatment-induced death.

The available *in vivo* evidence is also less detailed mechanistically than much of the cellular evidence. Several studies demonstrated altered tumor growth, metastasis, treatment sensitivity, or resistance, but dynamic autophagic flux and compartment-specific CTSB activity are more difficult to establish in tumor tissue. Changes in LC3-II, p62, or CTSB expression within excised tumors provide supportive evidence but do not independently prove the direction of flux or identify the responsible CTSB pool.

Human tumor-expression studies show associations between elevated CTSB and aggressive disease, but they do not determine whether CTSB supports autophagy, extracellular invasion, inflammation, or several processes simultaneously [34,35]. Expression-based prognostic evidence should therefore not be treated as direct support for pharmacological inhibition of lysosomal CTSB.

The literature also contains an imbalance between studies of broad lysosomal disruption and studies of selective CTSB inhibition. Severe autophagic blockade is more frequently produced by compounds affecting several cathepsins or lysosomal functions simultaneously.

This creates uncertainty regarding whether selective CTSB inhibition alone would produce sufficient loss of degradative capacity to generate a therapeutically useful effect.

Despite these limitations, the consistency of several mechanistic observations supports a genuine relationship. Preserved lysosomal CTSB activity can contribute to productive cargo degradation and metabolic adaptation [11]. Reduction of CTSB within a broader lysosomal defect can contribute to autophagic cargo accumulation [9,10,94–99]. Following lysosomal membrane permeabilization, cytosolic CTSB can participate in apoptosis or lysosome-dependent death [12,32,102–108]. The principal uncertainty concerns the conditions under which each function becomes dominant.

The current evidence should therefore be characterized as **mechanistically supportive but not universally definitive**. It supports CTSB as a context-dependent regulator of lysosomal degradation and cancer-cell fate, while demonstrating that pharmacological outcome is determined by localization, selectivity, protease redundancy, autophagic function, and treatment mechanism.

Future conclusions should be based on a hierarchy of evidence. The strongest studies should combine selective chemical inhibition with verified intracellular target engagement, assessment of other cathepsins, genetic depletion and rescue, dynamic autophagic-flux assays, compartment-specific CTSB measurements, and validation in multiple cancer models. Until such evidence becomes available, therapeutic claims should remain restricted to the specific experimental conditions in which they were demonstrated.

7.2 Therapeutic Opportunities and Risks of Cathepsin B Targeting

The therapeutic value of cathepsin B targeting depends on whether the intervention suppresses a tumor-supportive CTSB function without simultaneously disrupting a beneficial death pathway or essential lysosomal activity in normal tissues. The available evidence identifies several potential therapeutic opportunities, but each is associated with distinct pharmacological and biological risks.

One opportunity is the inhibition of extracellular or cell-surface-associated CTSB involved in matrix degradation and metastatic dissemination. In experimental breast cancer, pharmacological CTSB inhibition and RNA-interference-mediated CTSB reduction decreased collagen degradation and reduced pulmonary and bone metastasis [31]. Because this strategy primarily targets pericellular proteolysis, its therapeutic effect does not require inhibition of lysosomal autophagic degradation. An extracellularly restricted inhibitor could theoretically reduce invasion while limiting disruption of intracellular lysosomal functions. However, this approach would not be expected to suppress CTSB-supported metabolic recycling inside cancer cells.

A second opportunity is the inhibition of lysosomal CTSB in tumors that depend on efficient autophagic degradation. The pancreatic cancer study by Jiang et al. demonstrated that preservation of CTSB proteolytic activity promoted productive autophagic flux, glycolytic metabolism, proliferation, and tumor growth [11]. Constitutive CTSB loss also delayed pancreatic ductal adenocarcinoma progression in a genetically engineered mouse model [38]. Together, these findings provide a rationale for investigating lysosome-accessible CTSB inhibitors in metabolically dependent pancreatic tumors. Nevertheless, the direct therapeutic

effect of a highly selective lysosomal CTSB inhibitor has not yet been established in these models.

The potential metabolic benefit of CTSB inhibition is likely to be greatest when CTSB-supported lysosomal degradation represents a rate-limiting process. Tumors with high autophagic throughput may require continuous proteolytic recycling to maintain amino-acid availability and energy production. In such a setting, partial reduction of CTSB activity could increase metabolic stress or enhance sensitivity to treatments that impair nutrient acquisition. In contrast, tumors with substantial compensatory cathepsin activity may maintain sufficient lysosomal degradation despite CTSB inhibition.

A third therapeutic opportunity involves broad inhibition of tumor-associated cysteine cathepsins. The reversible multi-cathepsin inhibitor VBY-825 reduced tumor number and burden and increased apoptosis in a preclinical pancreatic islet-tumor model [74]. Such broad inhibitors may overcome redundancy between CTSB, CTSL, and related proteases more effectively than a selective CTSB inhibitor. However, these results validate the cysteine-cathepsin network rather than CTSB alone, and the greater reduction in collective lysosomal proteolysis may also increase toxicity in normal tissues.

Targeted delivery may improve the therapeutic window. A liposomal system displaying a lipidated CTSB inhibitor showed preferential association with tumor cells and tumor-associated macrophages in an experimental mammary cancer model [91]. This strategy illustrates how inhibitor exposure might be concentrated within tumor tissue while reducing systemic distribution. Nevertheless, selective tumor accumulation may result from several factors, including vascular permeability, nanoparticle uptake, and phagocytic-cell activity. Tumor localization must therefore be separated experimentally from selective engagement of the intended CTSB pool.

The principal risk of CTSB inhibition is that the enzyme is not uniformly tumor-supportive. Following lysosomal membrane permeabilization, cytosolic CTSB can contribute to treatment-induced cell death. In chronic myelogenous leukemia, imatinib promoted lysosomal CTSB release, BCR–ABL cleavage, and elimination of malignant cells. Pharmacological inhibition or genetic reduction of CTSB partially protected the cells, whereas increased CTSB expression enhanced treatment sensitivity [32]. Combining a CTSB inhibitor with a treatment that depends on lysosomal enzyme release could therefore antagonize rather than improve anticancer activity.

This risk is also relevant to treatments that induce oxidative stress, lysosomal destabilization, autophagy-associated membrane injury, or ferroptosis. In these settings, CTSB may function downstream as an execution or amplification factor. The detection of elevated CTSB expression in a tumor is insufficient to determine whether inhibition will be beneficial; it is necessary to establish whether the dominant enzyme pool supports lysosomal recycling, extracellular invasion, or treatment-induced death.

A second risk arises from inadequate inhibitor selectivity. CA-074Me can inhibit additional cysteine cathepsins under cellular conditions, whereas CA-074 may fail to reach intracellular CTSB efficiently during short exposures [13,75–77]. Broad inhibition may produce extensive lysosomal dysfunction that appears therapeutically effective in cultured cancer cells but does not represent a CTSB-specific effect. Conversely, inadequate intracellular access may cause a potentially useful target to appear pharmacologically irrelevant.

Physiological protease redundancy creates both protection and risk. Individual loss of CTSB can sometimes be compensated by related lysosomal proteases, reducing therapeutic efficacy. However, combined deficiency of cathepsins B and L produced severe neurodegeneration and early lethality in mice, demonstrating the potential consequences of excessive suppression of the lysosomal protease network [88]. These findings suggest that increased inhibitor breadth may overcome tumor resistance but simultaneously narrow the therapeutic window.

Systemic CTSB inhibition may also affect nonmalignant cells within the tumor microenvironment and other organs. In macrophages, CTSB contributed to trafficking of TNF- α -containing vesicles toward the plasma membrane [89]. Alteration of such immune-cell processes could modify inflammation, antitumor immunity, infection responses, or treatment toxicity. The final effect may differ between immunodeficient xenografts and immunocompetent tumor models.

Another therapeutic risk is the development of adaptive resistance. Prolonged CTSB inhibition may increase the abundance or activity of compensating cathepsins, select cancer-cell populations with reduced lysosomal dependency, or activate alternative nutrient-acquisition pathways. A strong response during acute treatment does not guarantee durable tumor control. Long-term experiments should therefore assess protease adaptation, metabolic recovery, and tumor regrowth after treatment withdrawal.

The ideal CTSB-directed strategy is unlikely to involve unrestricted systemic inhibition in all patients. A rational intervention would identify a tumor in which the relevant CTSB pool is catalytically active and functionally important, deliver the inhibitor selectively to that compartment, confirm target engagement, and demonstrate that the accompanying treatment does not require cytosolic CTSB for cancer-cell killing.

The intended therapeutic objective should also determine inhibitor design. Extracellularly restricted inhibitors may be most appropriate for reducing invasion and metastasis. Lysosome-directed inhibitors may be considered for tumors dependent on autophagic recycling. Tumor-targeted or conditionally activated inhibitors may reduce systemic toxicity, while broad cathepsin inhibition should be reserved for situations in which protease redundancy has been demonstrated and safety can be adequately controlled.

Overall, cathepsin B represents a potentially valuable but conditional therapeutic target. The opportunity lies in exploiting specific CTSB dependencies related to invasion, lysosomal metabolism, or tumor–stroma interactions. The principal risks are insufficient selectivity, physiological lysosomal toxicity, compensatory protease activity, immune effects, and interruption of CTSB-dependent cell-death pathways. Therapeutic success therefore requires mechanism-based selection of the tumor, treatment combination, inhibitor properties, and CTSB compartment.

7.3 Rational Combination Strategies and Biomarker-Guided Selection

Cathepsin B-directed treatment is more likely to be effective when incorporated into a mechanism-based combination rather than applied uniformly as monotherapy. The combination partner should be selected according to the relevant CTSB compartment, the functional role of autophagy, the degree of lysosomal dependency, and whether treatment-induced cell death requires cathepsin release. A combination can therefore be synergistic, ineffective, or antagonistic despite producing a similar reduction in CTSB activity.

The strongest direct evidence supporting a CTSB-associated combination strategy comes from ovarian cancer. Cisplatin stimulated an autophagic response that was compatible with continued cargo degradation, whereas bortezomib reduced CTSB expression, impaired autophagic flux, and increased cisplatin-induced cytotoxicity. Partial restoration of CTSB improved cargo clearance and cell survival, supporting the conclusion that suppression of a CTSB-containing lysosomal pathway contributed to the combination effect [9]. However, bortezomib also affected additional lysosomal enzymes and cannot be considered a selective CTSB inhibitor.

A similar therapeutic principle may apply when inhibition of an oncogenic pathway creates increased dependence on autophagic recycling. ERK-pathway inhibition increased protective autophagy in RAS-driven and pancreatic cancer models, while combined ERK and autophagy inhibition produced stronger antitumor effects than either strategy alone [68,69]. These studies validate treatment-induced autophagy dependency as a combination concept, but they do not establish that selective CTSB inhibition can reproduce the effects of broader autophagy inhibition. Direct testing with verified lysosomal CTSB inhibitors remains necessary.

Tumors with high basal autophagy–lysosome activity may represent another candidate setting. In pancreatic ductal adenocarcinoma, preserved CTSB activity supported autophagic flux, glycolysis, proliferation, and tumor growth [11]. Combining lysosomal CTSB inhibition with treatments that increase metabolic stress could therefore reduce nutrient recycling and narrow the adaptive capacity of the tumor. This remains a mechanistic proposal rather than an established therapeutic regimen because a selective CTSB inhibitor has not yet been validated in this model.

Broad late-stage autophagy inhibitors have also increased the effect of nutrient deprivation or chemotherapy in experimental cancer models. Oblongifolin C enhanced the cytotoxic consequences of nutrient deprivation, while elaiophyllin increased treatment-associated cancer-cell death [94,97,98]. These findings demonstrate that lysosomal degradation can be therapeutically disrupted, but the compounds affect several lysosomal and nonlysosomal targets. They cannot be used as direct evidence that selective CTSB inhibition will produce the same combination benefit.

Combination strategies must avoid treatments whose cytotoxicity depends on released cathepsin B. In chronic myelogenous leukemia, imatinib-induced lysosomal membrane permeabilization released CTSB into the cytosol, where it contributed to BCR–ABL cleavage and malignant-cell elimination. Pharmacological or genetic reduction of CTSB protected the cells partially from treatment [32]. A CTSB inhibitor combined with such a treatment could therefore reduce rather than improve therapeutic efficacy.

The same caution applies to lysosomotropic, oxidative-stress-inducing, and ferroptosis-inducing treatments when lysosomal permeabilization and cytosolic cathepsin activity form part of the death mechanism. Before combination treatment is selected, experiments should establish whether CTSB remains confined within lysosomes or is released into the cytosol and whether CTSB inhibition increases or decreases long-term cancer-cell survival.

Extracellularly restricted CTSB inhibition represents a separate combination opportunity. Such an inhibitor could be combined with treatments directed against primary-tumor growth while specifically reducing pericellular proteolysis, invasion, and metastatic dissemination.

Because extracellular CTSB inhibition is not expected to produce strong autophagic blockade, the success of this strategy should be evaluated using matrix degradation, invasion, and metastasis endpoints rather than LC3-II or p62.

Patient and model selection should not be based on total CTSB expression alone. The most relevant variable is the abundance and accessibility of catalytically active CTSB within the compartment targeted by the treatment. High expression may represent inactive procathepsin B, extracellular enzyme, lysosomal enzyme, or CTSB derived from stromal cells. These pools do not predict the same therapeutic response.

Candidate biomarkers for lysosomal CTSB-directed treatment include:

- high mature lysosomal CTSB activity;
- elevated basal autophagic flux and lysosomal proteolysis;
- dependence on autophagy for metabolism or treatment resistance;
- limited compensatory cathepsin activity;
- evidence that CTSB reduction impairs cargo degradation;
- absence of a treatment mechanism requiring cytosolic CTSB release.

These are **proposed mechanistic biomarkers**, not clinically validated selection criteria. Their usefulness must be tested prospectively in several tumor models and eventually in human tumor material.

Activity-based probes may help identify catalytically active CTSB and confirm pharmacological target engagement. Nevertheless, detection of tumor-associated activity does not independently demonstrate that the measured enzyme is lysosomal or that the tumor depends on it. Activity imaging should therefore be combined with compartment-specific analysis, autophagic-flux measurements, and functional intervention experiments.

Table 3. Mechanism-based framework for cathepsin B-directed combination strategies

Therapeutic context	Proposed combination logic	Required evidence before combination	Principal risk	Evidence status and principal limitation
Treatment induces cytoprotective autophagy	Reduce lysosomal recycling to increase treatment sensitivity	Productive treatment-induced flux, CTSB contribution to degradation, and enhanced killing after CTSB suppression	General lysosomal toxicity or lack of CTSB dependency	Supported indirectly and by the bortezomib–cisplatin model [9]
Tumor has high metabolic dependence on autophagy	Combine CTSB inhibition with metabolic or oncogenic-pathway stress	High basal flux, active lysosomal CTSB, metabolic decline after CTSB suppression	Compensation by other cathepsins	Strong biological rationale in pancreatic cancer, but selective pharmacological validation is absent [11,68,69]
Extracellular CTSB promotes invasion	Combine extracellularly restricted inhibition with tumor-directed therapy	Pericellular CTSB activity and functional matrix-degradation assays	Limited effect on primary-tumor metabolism	Preclinical antimetastatic support [30,31]
Broad cathepsin redundancy limits selective inhibition	Combine or replace selective CTSB targeting with controlled multi-cathepsin inhibition	Demonstrated compensation and tolerable normal-tissue effects	Severe physiological lysosomal toxicity	Preclinical support with multi-cathepsin inhibitors [73,74,88]
Anticancer treatment induces lysosomal membrane permeabilization	Preserve rather than inhibit death-promoting cytosolic CTSB	Demonstration that CTSB release precedes and promotes death	CTSB inhibition may antagonize treatment	Strong mechanistic warning in CML and supporting models [32,102–108]
Drug undergoes lysosomal sequestration	Target lysosomal drug storage together with the resistance pathway	Measurement of drug distribution, lysosomal function, and target availability	Lysosomal damage may increase toxicity without restoring drug action	Context-specific evidence from sunitinib resistance [99]

Note: The proposed strategies represent a mechanistic framework rather than clinically validated treatment recommendations. A greater effect of two treatments in combination does not independently establish pharmacological synergy. Synergy should be evaluated using concentration matrices and an appropriate quantitative interaction model. CTSB inhibition should be considered only after confirming the relevant enzyme compartment, catalytic target engagement, autophagic function, and final effect on cancer-cell fate.

Abbreviations: CML, chronic myelogenous leukemia; CTSB, cathepsin B; CTSD, cathepsin D; CTSL, cathepsin L; LMP, lysosomal membrane permeabilization.

The most informative preclinical combination experiments should use treatment matrices rather than one dose of each compound. They should measure target engagement, dynamic autophagic flux, lysosomal integrity, related cathepsins, short-term cell death, clonogenic recovery, and tumor regrowth. Synergy should be quantified rather than inferred from a greater effect of two drugs compared with either treatment alone.

Validation should progress from cancer-cell cultures to organoids, patient-derived models, and immunocompetent animal systems. This sequence is important because stromal cells and macrophages contribute CTSB activity and may modify both therapeutic response and toxicity. Pharmacodynamic measurements should confirm that the intended CTSB compartment is inhibited at an exposure that remains tolerable.

Overall, combination therapy should be guided by a mechanistic decision: suppress CTSB when it supports lysosomal recycling or extracellular invasion, but preserve its activity when treatment-induced cytosolic release contributes to cancer-cell death. Biomarker-guided selection is therefore essential for converting the context-dependent biology of CTSB into a rational therapeutic strategy.

7.4 Future Experimental and Translational Priorities

Future research should move beyond demonstrating associations between changes in CTSB expression, autophagy markers, and cancer-cell viability. The central experimental objective should be to determine whether selective inhibition of a defined cathepsin B pool directly changes lysosomal proteolysis, dynamic autophagic flux, and cancer-cell fate. Achieving this objective requires an integrated pharmacological, genetic, biochemical, and functional strategy.

The first priority is the development and validation of inhibitors that combine high biochemical selectivity with access to the intended cellular compartment. A candidate lysosomal CTSB inhibitor should enter cancer cells, accumulate within endolysosomal compartments, remain active under acidic conditions, and inhibit mature CTSB without substantially suppressing cathepsins L, S, or other related enzymes. Biochemical selectivity alone is insufficient because intracellular conversion, pH, redox conditions, and inhibitor concentration may alter target specificity [13,75–77].

At least two structurally distinct inhibitors should be used whenever possible. Reproduction of the same phenotype with unrelated chemical scaffolds reduces the probability that the result is caused by a compound-specific off-target effect. Competition experiments with activity-based probes should confirm inhibition of catalytically active CTSB in intact cells and determine whether related cysteine cathepsins remain functional [78–81].

Compartment-specific target engagement must also be established. Whole-cell CTSB activity does not reveal whether the inhibited enzyme is lysosomal, extracellular, or cytosolic. Lysosomal fractionation, organelle-directed probes, imaging, and comparison between cell-permeable and extracellularly restricted inhibitors can help identify the pharmacologically

affected pool. This distinction is essential because the same intervention may reduce lysosomal recycling, extracellular invasion, or cytosolic death signaling depending on its distribution.

Genetic validation should complement rather than replace pharmacological inhibition. Acute CTSB knockdown can reduce the opportunity for long-term adaptation, whereas stable knockout models can identify chronic compensatory mechanisms. Rescue with wild-type CTSB should determine whether the phenotype can be reversed. Comparison with a catalytically inactive but correctly localized CTSB variant would further distinguish dependence on enzymatic activity from effects caused by the presence, trafficking, or molecular interactions of the protein.

Related lysosomal proteases should be measured throughout the experiment. Cathepsins L and D are particularly important because changes in their expression, maturation, or activity may compensate for CTSB loss or contribute independently to cargo degradation. Measurements should be performed shortly after inhibition and after prolonged exposure to distinguish immediate protease redundancy from later adaptive compensation [38,42,88].

Autophagy should be evaluated dynamically and at multiple pathway stages. A minimum assay panel should include LC3-II turnover in the presence and absence of a late-stage inhibitor, p62 protein and mRNA measurements, and at least one additional cargo-degradation or tandem-reporter assay. Fusion, lysosomal acidity, proteolytic capacity, and membrane integrity should be evaluated separately. This design is required to distinguish increased autophagosome formation, defective fusion, post-fusion proteolytic failure, and lysosomal membrane permeabilization [1,57–61].

The temporal sequence of events should be defined using several treatment durations. Early measurements should determine whether CTSB inhibition precedes changes in lysosomal degradation. Later measurements should establish whether cargo accumulation, metabolic stress, compensatory protease activity, membrane permeabilization, or cell death develops subsequently. Without temporal analysis, a late secondary loss of CTSB activity may be incorrectly identified as the initiating mechanism.

Cellular outcome should be evaluated using more than short-term viability assays. Apoptosis, ferroptosis, lysosome-dependent death, proliferation, clonogenic recovery, and long-term regrowth should be distinguished experimentally. Functional interference with autophagy should determine whether the altered flux is cytoprotective, cytotoxic, cytostatic, or nonprotective under the specific treatment condition.

Cancer-model selection should include biologically contrasting systems. Models with high basal lysosomal CTSB activity and autophagic dependency should be compared with models displaying low CTSB activity or effective cathepsin compensation. At least one nonmalignant cell model should be included to estimate the therapeutic window and determine whether the intervention selectively affects tumor-associated lysosomal demand.

Three-dimensional organoids, patient-derived tumor models, and co-culture systems should be used after initial mechanistic validation. These models can preserve aspects of tumor heterogeneity, extracellular matrix organization, nutrient gradients, and interactions with macrophages or other stromal cells that are absent from conventional monocultures.

However, complex models should not replace detailed mechanistic experiments because they may make compartment-specific target attribution more difficult.

Validation in immunocompetent animal models is particularly important for systemic CTSB inhibition. Cathepsin B activity in macrophages and other host cells can contribute to tumor progression, inflammation, and treatment response [36,37,89]. Immunodeficient xenografts may demonstrate direct tumor-cell effects but cannot fully evaluate immune consequences or host-derived CTSB activity.

Targeted inhibitor delivery represents a promising approach to improving compartment and cell-type selectivity. A 2024 study developed antibody–peptide inhibitor conjugates that delivered covalent inhibitors to selected cancer-cell contexts, blocked cysteine-cathepsin activity, and showed efficacy in cellular and animal models [113]. This platform demonstrates the feasibility of restricting cathepsin inhibition to selected cells, but it does not yet establish selective inhibition of lysosomal CTSB-dependent autophagy.

Pharmacokinetic and pharmacodynamic studies should determine circulating exposure, tissue distribution, intracellular accumulation, duration of target inhibition, and recovery after treatment withdrawal. Pharmacodynamic biomarkers should demonstrate inhibition of the intended CTSB pool at doses that do not cause unacceptable lysosomal dysfunction in the nervous system, liver, kidney, immune system, or other normal tissues.

Biomarker development should focus on functional dependency rather than expression alone. Candidate biomarkers include mature CTSB activity, high basal autophagic flux, strong lysosomal proteolysis, metabolic sensitivity to CTSB depletion, and limited compensatory cathepsin activity. These biomarkers should be tested prospectively to determine whether they predict response to CTSB inhibition rather than merely correlate with aggressive disease.

Combination studies should include both efficacy and antagonism testing. CTSB inhibition should be combined with treatments that produce metabolic stress or cytoprotective autophagy only after demonstrating that lysosomal CTSB supports the protective response. Conversely, treatments that induce lysosomal membrane permeabilization should be examined carefully because CTSB inhibition may suppress therapeutically beneficial apoptosis or ferroptotic damage.

Table 4. Minimum evidence framework for attributing an autophagy phenotype specifically to cathepsin B modulation

Experimental level	Minimum requirement	Purpose
Pharmacological identity	At least one validated inhibitor, preferably supported by a second chemical scaffold	Reduces dependence on compound-specific off-target effects
Cellular target engagement	Direct measurement of mature CTSB catalytic inhibition in intact cells	Confirms that the intended enzyme is actually inhibited
Compartment identification	Evidence that the affected CTSB pool is lysosomal, extracellular, or cytosolic	Connects enzyme inhibition with the relevant biological function
Protease selectivity	Measurement of cathepsins L, D, S, and other relevant hydrolases	Distinguishes CTSB-specific effects from broad lysosomal inhibition
Genetic validation	Acute depletion or knockout with wild-type and, when feasible, catalytic-mutant rescue	Tests causal and catalytic dependence
Dynamic autophagic flux	LC3-II turnover plus p62 or another cargo assay and a complementary reporter	Distinguishes productive flux from intermediate accumulation
Lysosomal function	Separate assessment of fusion, acidity, proteolysis, and membrane integrity	Identifies the precise stage of pathway failure
Temporal sequence	Multiple early and late treatment time points	Determines whether CTSB alteration precedes the phenotype
Functional consequence	Apoptosis, ferroptosis, proliferation, clonogenic survival, and tumor growth where relevant	Establishes the biological meaning of the autophagy change
Model diversity	Multiple cancer models, a nonmalignant comparator, and advanced three-dimensional or in vivo validation	Tests generalizability and therapeutic selectivity
Pharmacokinetics and safety	Tissue exposure, target inhibition, normal-organ lysosomal function, and reversibility	Defines the potential therapeutic window
Biomarker validation	Prospective testing of active CTSB and lysosomal-dependency markers	Supports rational selection of responsive tumors

No single assay can establish that selective CTSB inhibition alters autophagy. A convincing conclusion requires agreement between pharmacological target engagement, genetic validation, dynamic flux measurements, lysosomal-function assays, and the final biological outcome. Failure at any one level should narrow the interpretation rather than being ignored.

The most decisive future experiment would compare a selective lysosome-accessible CTSB inhibitor with genetic CTSB depletion across several cancer models displaying different degrees of autophagy dependency. The study should measure other cathepsins, perform dynamic flux and lysosomal assays, include rescue with active CTSB, and evaluate metabolism, treatment response, tumor growth, and normal-tissue toxicity.

Such an experimental design would determine whether CTSB possesses a therapeutically exploitable nonredundant role in terminal autophagic degradation or whether substantial anticancer activity requires broader disruption of the lysosomal protease network. Resolving this question is essential before lysosomal CTSB inhibition can progress from a mechanistically plausible concept to a biomarker-guided anticancer strategy.

Figure 6. Biomarker-guided decision framework for pharmacological targeting of cathepsin B in cancer

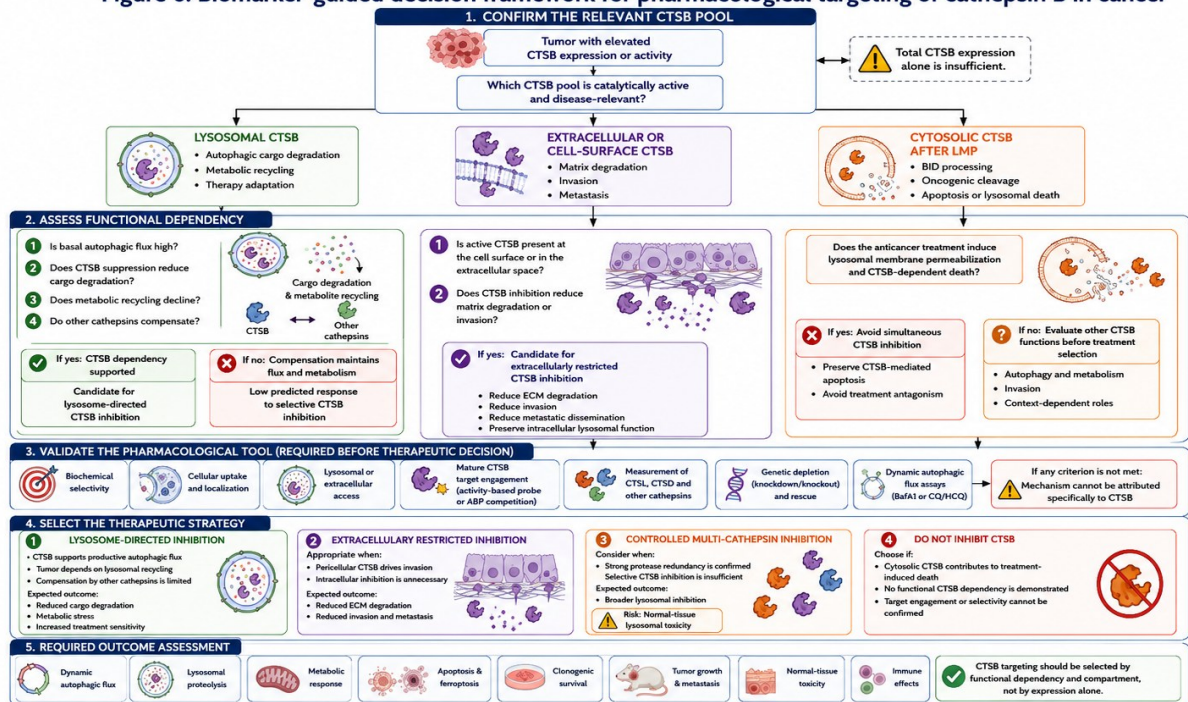


Figure 6. Biomarker-guided decision framework for pharmacological targeting of cathepsin B in cancer. Selection of a cathepsin B-directed strategy requires identification of the catalytically active and biologically relevant CTSB pool. Lysosomal CTSB inhibition may be appropriate when the enzyme supports productive autophagic degradation and tumor metabolism and when compensation by other lysosomal proteases is limited. Extracellularly restricted inhibition may reduce matrix degradation and metastatic invasion while preserving intracellular lysosomal function. In contrast, CTSB inhibition should be avoided when treatment-induced lysosomal membrane permeabilization releases cytosolic CTSB that contributes to cancer-cell death. Pharmacological selectivity, compartment-specific target engagement, genetic validation, dynamic autophagic-flux assessment, and functional outcome testing are required before therapeutic conclusions are made. Conceptual synthesis based on references [9–13,30–32,38,42,57,68,69,73–81,88–92,99,102–110]. Created by the author with the assistance of artificial intelligence and Biorender.com

Figure 6. Biomarker-guided decision framework for pharmacological targeting of cathepsin B in cancer

8. Conclusion

8.1 Integrated Conclusions

This thesis critically evaluated how pharmacological targeting of lysosomal cathepsin B influences autophagy in cancer cells. The reviewed evidence demonstrates that cathepsin B forms an important mechanistic connection between lysosomal proteolysis, autophagic cargo degradation, metabolic adaptation, and cancer-cell fate. However, the biological consequence of altering CTSB activity is not uniform and cannot be predicted solely from changes in enzyme expression or autophagy-associated markers.

Within intact lysosomes and autolysosomes, mature cathepsin B contributes to terminal proteolysis of material delivered through autophagic and endocytic pathways. Its function occurs within a broader network of cathepsins and other lysosomal hydrolases, meaning that CTSB is important but not universally indispensable for bulk lysosomal degradation. The extent to which reduced CTSB activity impairs autophagic clearance depends on the identity and quantity of the substrates, basal lysosomal demand, residual activity of other proteases, and capacity for compensatory adaptation.

The available evidence most clearly supports a CTSB-dependent contribution to productive autophagic flux in pancreatic ductal adenocarcinoma. Preservation of CTSB activity through CTSB-dependent regulation promoted lysosomal cargo degradation, glycolytic metabolism, proliferation, and tumor growth [11]. This study demonstrates that lysosomal CTSB can support a tumor-promoting autophagy–metabolism axis. Nevertheless, direct pharmacological validation using a highly selective and lysosome-accessible CTSB inhibitor remains absent.

Evidence for late-stage autophagic blockade is more extensive but less specific pharmacologically. Bortezomib, ADCX, oblongifolin C, obatoclax, elaiophyllin, and sunitinib were associated with reduced CTSB expression or activity, impaired cargo clearance, and accumulation of LC3-II, p62, or autophagic compartments [9,10,94–99]. However, these compounds also altered additional cathepsins, intracellular signaling, lysosomal acidification, membrane integrity, autophagosome–lysosome fusion, or drug sequestration. Most therefore demonstrate CTSB-associated lysosomal dysfunction more convincingly than selective inhibition of CTSB alone.

Among these studies, the bortezomib model provides comparatively strong evidence for a functional CTSB contribution because restoration of CTSB partially improved p62 degradation and cancer-cell survival [9]. Even in this model, the concurrent reduction of additional lysosomal enzymes prevents exclusive attribution of the phenotype to CTSB. The current literature consequently supports the conclusion that reduced CTSB can contribute to terminal degradation failure, but it does not establish that selective CTSB inhibition is sufficient to cause complete autophagic blockade in every cancer type.

A central conclusion of the thesis is that autophagosome accumulation must not be equated with autophagy induction. Increased LC3-II, p62, fluorescent puncta, or autophagic vacuoles may indicate increased formation, reduced clearance, or simultaneous changes at both stages. Productive autophagic flux can be established only through dynamic measurements demonstrating continued lysosomal delivery and cargo degradation [1,54,57–61]. This

distinction is particularly important for CTSB because its principal lysosomal function occurs at the terminal degradative stage rather than during autophagosome initiation.

The final cellular outcome of impaired flux is also context-dependent. Reduced lysosomal recycling may deprive metabolically dependent cancer cells of substrates, increase accumulation of damaged material, and enhance treatment sensitivity. Conversely, lysosomal dysfunction may contribute to resistance by sequestering anticancer drugs or activating adaptive pathways, as demonstrated in sunitinib-resistant renal-cell carcinoma [99]. A reduction in autophagic flux is therefore a mechanistic observation rather than an automatic indicator of therapeutic benefit.

Cathepsin B localization provides a second major determinant of biological outcome. Lysosome-confined CTSB principally supports controlled proteolysis. Extracellular and cell-surface-associated CTSB may promote matrix degradation and invasion. After lysosomal membrane permeabilization, CTSB can enter the cytosol and participate in oncogenic-protein cleavage, BID-associated mitochondrial signaling, apoptosis, ferroptotic damage, or other forms of lysosome-dependent cell death [12,30–32,100–110].

This localization-dependent behavior explains why CTSB inhibition can produce opposite therapeutic effects. Suppression of lysosomal CTSB may weaken metabolic recycling in a tumor dependent on productive autophagic flux. In contrast, inhibition of cytosolic CTSB may protect malignant cells when an anticancer treatment relies on lysosomal membrane permeabilization and cathepsin-mediated death signaling. The effect of CTSB inhibition must therefore be evaluated in relation to the treatment mechanism and the enzyme compartment affected.

The analysis also demonstrates that the evidence base on CTSB targeting includes mechanistically different approaches. Direct catalytic inhibition, indirect pharmacological alteration of CTSB expression or maturation, genetic depletion used for mechanistic validation, and CTSB-responsive drug-delivery systems cannot be interpreted as equivalent approaches. Cathepsin B-activatable prodrugs and nanocarriers exploit proteolytic activity to release a therapeutic payload; they do not test the consequences of reducing lysosomal CTSB function. Clear separation of these approaches is essential for accurate interpretation.

Pharmacological selectivity remains a major limitation. CA-074 can display relatively strong biochemical selectivity but may enter cells inefficiently during short exposures. CA-074Me has better cellular access but may inhibit cathepsin L and other cysteine proteases under defined conditions [13,72,75–77]. Findings based on either inhibitor require direct confirmation of intracellular CTSB target engagement and measurement of related cathepsins before a cellular phenotype is assigned specifically to CTSB.

Protease redundancy further restricts therapeutic interpretation. Cathepsin L and other hydrolases may preserve part of the lysosomal degradative capacity after CTSB inhibition, particularly during chronic genetic loss or prolonged treatment [38,42,88]. Broad multi-cathepsin inhibition may produce stronger autophagic blockade but carries a greater risk of disrupting essential lysosomal functions in normal tissues. The most therapeutically suitable tumors may therefore be those in which CTSB performs a rate-limiting or relatively nonredundant role.

The available evidence is best characterized as **mechanistically supportive but not universally definitive**. It establishes that CTSB can support productive autophagic degradation, that its reduction can contribute to lysosomal cargo-clearance failure, and that its cytosolic release can participate in cancer-cell death. What remains unresolved is whether selective pharmacological inhibition of lysosomal CTSB alone can consistently produce a clinically useful autophagy phenotype across defined cancer subgroups.

A convincing future demonstration will require selective lysosome-accessible inhibitors, direct verification of cellular target engagement, measurement of related cathepsins, dynamic autophagic-flux analysis, genetic depletion and rescue, compartment-specific CTSB assessment, and evaluation of metabolism, treatment response, and long-term tumor growth. These experiments must also establish safety in normal tissues and determine whether a given combination treatment depends on CTSB-mediated death signaling.

The principal conclusion of this thesis is therefore not that cathepsin B should be universally inhibited in cancer. Instead, CTSB represents a conditional pharmacological target whose value depends on enzyme localization, tumor lysosomal dependency, protease compensation, autophagic function, inhibitor selectivity, and treatment mechanism. Therapeutic targeting is most rational when a tumor-specific CTSB dependency has been experimentally demonstrated and the affected enzyme pool can be inhibited without suppressing beneficial cell-death pathways or essential physiological lysosomal activity.

8.2 Final Perspective

The relationship between cathepsin B and autophagy in cancer cannot be reduced to a simple model in which increased CTSB is always harmful and its inhibition is always therapeutically beneficial. Cathepsin B performs different functions according to its molecular form, cellular localization, lysosomal environment, and the biological demands of the tumor. The same enzyme may support intracellular recycling within intact lysosomes, promote invasion in the extracellular compartment, or contribute to cancer-cell death after release into the cytosol.

This functional diversity explains much of the apparent disagreement among experimental studies. Findings that initially appear contradictory often investigate different CTSB pools, pharmacological interventions, cancer models, or stages of the autophagy–lysosome pathway. A compound that reduces CTSB expression indirectly cannot be considered equivalent to a selective catalytic inhibitor, and a treatment that causes broad lysosomal damage cannot establish the effect of CTSB inhibition alone.

The most important conceptual requirement is therefore precise mechanistic definition. Future studies must determine which CTSB pool is affected, whether catalytic target engagement has occurred, whether related proteases compensate, and whether autophagic cargo is successfully degraded. Changes in LC3-II, p62, or autophagosome abundance should be interpreted within this complete experimental framework rather than used as isolated evidence of autophagy induction or inhibition.

From a therapeutic perspective, selective inhibition of lysosomal CTSB may be valuable in tumors that depend strongly on CTSB-supported proteolysis and metabolic recycling. Extracellularly restricted inhibition may offer a different opportunity by reducing invasion and metastasis. In contrast, CTSB inhibition should be avoided when cytosolic enzyme release contributes to the mechanism of treatment-induced cancer-cell death.

The future clinical relevance of CTSB targeting will consequently depend on biomarker-guided selection rather than universal application. Catalytically active CTSB, tumor dependence on autophagic recycling, limited protease compensation, and accessibility of the relevant enzyme compartment may together identify responsive tumors. These characteristics require prospective experimental validation before they can be used clinically.

Overall, cathepsin B should be viewed as a compartment-dependent and context-dependent regulator of cancer biology. Its pharmacological value lies not simply in inhibiting the enzyme, but in identifying the specific tumors, cellular compartments, and treatment conditions in which modulation of CTSB produces a predictable therapeutic advantage.

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