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Engaging the Lysosome and Lysosome-Dependent Cell Death in Cancer

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Abstract: While patient-specific targeting of cellular growth and viability pathways dominates current approaches in anti-cancer therapeutics development, appreciation for the strategy of targeting transformation-dependent alterations in cellular organelle structure and function continues to grow. Here we discuss the lysosome as an anti-cancer target, highlighting its role as a key mediator of cell death. As the major degradative compartment of the cell, the lysosome houses dozens of destructive enzymes and is responsible for the breakdown of both internal and external molecules and particles; however, until relatively recently the contribution of the lysosome to cellular death mechanisms has been largely overlooked.

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Renewed interest in the therapeutic potential of lysosomal rupture to combat cancer has led to development of lysosome-disrupting agents that induce lysosomal membrane permeabilization (LMP), cathepsin protease release, and subsequent lysosome-dependent cell death (LDCD), now distinguished as a bona fide cell death process. Here, we present the basic biology, structure, and function of the lysosome, with particular emphasis on the transformation-associated alterations that sensitize cancer cell lysosomes to membrane rupture. We further describe the lysosome's role in cell death and comprehensively outline emerging therapeutic strategies that exploit lysosomes for the treatment of a variety of malignancies.

Keywords: cancer therapeutic targeting; cationic amphiphilic drugs; lysosomal membrane permeabilization; lysosome; lysosome-dependent cell death

INTRODUCTION

Using subcellular fractionation based purely on biochemical criteria, de Duve and colleagues (1955) made the serendipitous discovery of the lysosome (1, 2), an achievement deemed worthy of the Nobel Prize in Physiology (1974). Upon observation that liver-derived acid phosphatase exhibited latent activity following homogenization, it was deduced that a membrane-bound structure must normally sequester it, and potentially other degradative hydrolases, from their substrates (2, 3). Indeed, the lysosome harbors some 60 lytic enzymes (4) capable of degrading proteins, nucleic acids, polysaccharides, and lipids. Subsequent investigations determined that lysosomes serve as the terminal compartment for the degradation of extracellular materials taken up by endocytosis and phagocytosis and the digestion of intracellular constituents isolated during autophagy (5, 6). We now appreciate that lysosomes are more than cellular refuse depots; they are fundamental components of dynamic physiologic processes such as plasma membrane repair, bone and tissue remodeling, matrix degradation, inflammatory responses, antigen presentation, cholesterol homeostasis, nutrient sensing and metabolism, cell signaling, growth factor recycling, and programmed cell death (7–23). These processes differentially rely on fusion with endocytic vesicles or the regulated release of lysosomal hydrolases into the cytosol via lysosomal membrane permeabilization (LMP) or into the extracellular space via lysosomal exocytosis. A summary of the lysosome's various functions is illustrated in Figure 1.

Of note are observations that the quantity, composition, and complement of lysosomal hydrolases are often augmented with cancer pathologies. Along these lines, lysosomal heparanase and cathepsins promote cancer cell proliferation, angiogenesis, and metastasis, suggesting that these and other lysosomal enzymes are of potential clinical significance (24, 25). The therapeutic implications of lysosomal hydrolases were recognized decades ago, with seminal investigations describing enhanced activities of lysosomal enzymes in solid tumors as compared to their tissues of origin, with specific enzymes (i.e., beta-glucuronidase) favoring tumor cell invasiveness (26). Perhaps it is not surprising then that cancer cells often exhibit an expansion of the lysosomal compartment (24, 27, 28), a feature that would enhance tumor aggressiveness. However, such distinction may also provide a rational basis for therapeutic intervention. Cancer-associated lysosomes

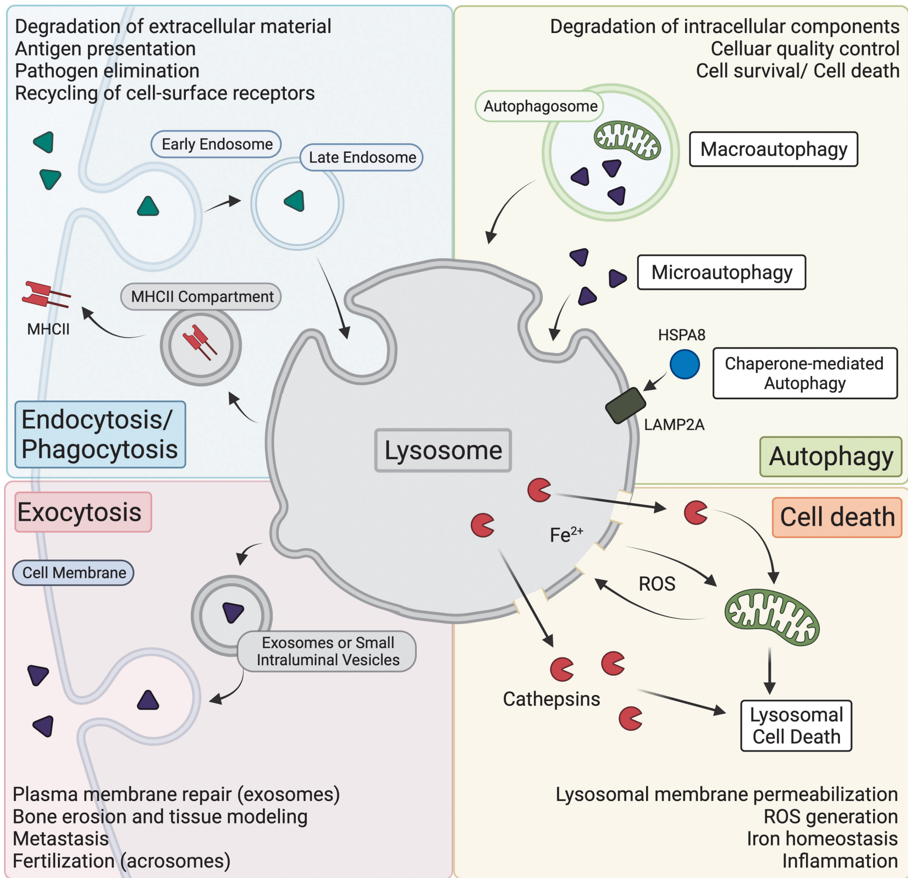

Figure 1. Functions of the lysosome. Lysosomes regulate cell function by internalizing and degrading pathogens, receptors, cellular debris, etc. via endocytosis and phagocytosis (upper left) and autophagy (upper right). They also transmit materials to the cell surface and extracellular space by exocytosis (lower left). Lysosomal membrane permeabilization resulting from various stimuli (e.g., reactive oxygen species (ROS) and iron accumulation) promotes cathepsin release and subsequent lysosomal cell death (lower right). Figure created with BioRender.com (adapted with permission from ref 163).

are more fragile than their normal counterparts due in some measure to increases in hydrolytic enzymes and fundamental changes in the composition of the lysosomal membrane (29–31). Based on the concept that the lysosome may represent a 'suicide-bag' as first proposed by de Duve (32), the instability of cancer-associated lysosomes may lend to enhanced cellular susceptibility to LMP, coincident release of destructive hydrolases into the cytosol and ultimate cell demise by either apoptotic or non-apoptotic cell death mechanisms.

Herein, we review the fundamentals of lysosome physiology, composition, and function in cell death, and connect cancer-associated changes in the expression and activity of lysosomal components with a particular focus on the therapeutic opportunities they may provide for breast and other tumor types.

LYSOSOMAL STRUCTURE, DISTRIBUTION, AND IDENTIFICATION

Lysosomes are typically less than 1 µm in diameter and contribute up to 0.5% of the total intracellular volume of many eukaryotic cells, although this may vary depending upon cell type (i.e., macrophages), energetic state, or degradative requirements (33–35). Unlike other organelles, lysosomes cannot be identified based on uniform morphologic criteria, as there is significant variation in their size, architecture, and morphology depending upon nutrient availability, for example, autophagy (36–39). Significant augmentation of lysosome volume, abundance, and structure also occurs during certain pathologic states, for example, lysosomal storage diseases (40) and cancer (24, 41), or following experimental manipulations that inhibit enzymatic digestion such as overloading with non-physiologic substrates such as sucrose (42), administration of cationic amphiphilic drugs (CADs (43–45)), or treatment with aminoglycoside antibiotics (46). Interestingly, lysosomes at peripheral locations can partially change their intracellular pH (47, 48), a feature that may be co-opted by some cancer cells to facilitate constitutive mTOR signaling (38) or modulate extracellular acidity to enhance invasion (49, 50).

By electron microscopy, lysosomes are identifiable as either tubular or spherical membrane-bound structures with cores of variable densities, amorphous granular material, or membrane whorls (34, 51). Biochemically, lysosomes are defined by the presence of multiple hydrolytic enzymes (32) which may vary between tissue type (51) and pathology (25, 40). Lysosomes may be distinguished from endosomes by their pH, calcium content, abundance of lysosomal-associated membrane proteins LAMP-1 and LAMP-2, and lack of mannose-6-phosphate receptors (34, 52–54). Fluorescent dyes that accumulate in acidic vesicles—such as LysoTracker Red and Acridine Orange—effectively label lysosomes, however other acidic vesicles such as endosomes and autophagosomes may be concomitantly labeled to varying degrees (55).

LYSOSOME COMPOSITION

Lysosomes can degrade a vast array of structurally diverse macromolecules into their constituent components. Following degradation, substances either diffuse or are transported out of the lysosome into the cytosol where they become fuel for metabolism or substrates for biosynthetic pathways (5). This dynamic recycling process requires the coordinated action of the lysosomal acid hydrolases with integral, peripheral, and transiently-associated proteins as discussed in the following sections.

Lysosomal hydrolases

To achieve efficient breakdown of complex substrates, lysosomes contain several acid hydrolases such as proteases, glycosidases, nucleases, sulfatases, and lipases. In addition to the degradation of material delivered via endosomes, phagocytic

vesicles, and autophagosomes, lysosomal hydrolases are involved in diverse processes such as pro-protein and antigen processing, degradation of extracellular matrix, stimulation of angiogenesis, and the initiation of cell death (13, 56–61).

Chief among the acid hydrolases are the aspartic, serine, and cysteine proteases, with the most widely studied being the cathepsins (62). While cathepsins are most recognized for their activity within the lysosomal compartment, a number of studies have indicated their localization to any vesicle along the endocytic pathway (early and late endosomes, phagosomes), within the nucleus or cytosol, at the cell surface, or secreted into the extracellular matrix, depending on physiologic or pathologic state (61–64). Cathepsins are synthesized as inactive precursors that are then processed to their mature and active form by proteolytic removal of the N-terminal propeptide (65). Removal of the propeptide may occur by autolysis within acidic lysosomes or by activation of other proteases in a chain-like reaction (66–70) and may be enhanced in the presence of glycosaminoglycans or polysaccharides (71–74). While most cathepsins become destabilized at neutral pH, several interacting partners such as heparin and catalase may prolong cathepsin activity by promoting structural integrity and inhibiting peroxidation (75, 76).

Originally considered to function only within the lysosome in general protein turnover, it has become exceedingly clear from gene knockout models that cathepsins have non-redundant and diverse functions and may be expressed ubiquitously in a tissue-specific or even context-specific manner (77–82). The diversity of this class of proteases is beyond the scope of this review and has been extensively examined elsewhere (63, 83–85). Notably, cysteine proteases B, L, S, X, and K, as well as aspartic cathepsin D, have all been implicated to varying degrees in cancer progression. Along these lines, cathepsins in cancer cells are often translocated to the plasma membrane along with pH regulators such as v-ATPases and Na^+/H^+ exchangers (86), where they associate with microdomains or are secreted in an active form (87). In this respect, cancer cells effectively exploit cathepsins to remodel the extracellular environment to potentiate invasion and metastasis (61, 88–93). Alternatively, infiltrating macrophages may supply cathepsins to stimulate angiogenesis and promote the growth and invasion of associated tumor cells (94).

Cathepsins may be specifically regulated by interactions with endogenous inhibitors, including cytosolic stefins, extracellular cystatins, and kininogens (63, 93). As such, blunted cystatin often accompanies enhanced cathepsin levels during the acquisition of invasive capacity (95). More recently, cathepsins have been implicated in the development of intrinsic therapeutic resistance and adaptive responses to treatment (93, 96–98).

Membrane-associated proteins and lipids

The lysosomal membrane contains more than one hundred proteins, with LAMP-1 and -2 comprising nearly 50% of the total protein content (99). The oligosaccharide side chains on LAMPs and LIMPs (lysosomal integral membrane proteins) form a thick polysaccharide coat, or glycocalyx, that lines the inner surface of the lysosomal membrane to ensure protection of sensitive lysosomal and extralysosomal substrates from degradative hydrolases (100, 101). In addition to ensuring compartmentalization of acid hydrolases and maintaining structural integrity,

peripheral and integral membrane-associated proteins are vital to lysosomal (LAMP-1) and the plasma membrane (RABs and SNAREs) trafficking, transport of ions and soluble substrates (cation channel mucolipin1, chloride channel CLCN7, protein transporter LAMP-2A, amino acid transporter LAAT1), and nutrient sensing (v-ATPase), as previously reviewed (52, 102–106). In addition, lysosomes may contain multiple internal vesicles that harbor their own unique complement of proteins and lipids (107, 108), imparting further functional diversity. Given this diversity and the propensity for alterations during cancer, it is worth detailing a few key membrane constituents. For a more in-depth discussion, the reader is referred to several excellent topical reviews (108–113).

Several observations suggest that LAMPs may contribute to the fragility of cancer-associated lysosomal membranes. Oncogenic transformation of fibroblasts is accompanied by a decrease in LAMP expression, redistribution of lysosomes to the cell periphery, and increased sensitivity to lysosomal cell death and to agents that induce LMP (114). Conversely, LAMP overexpression was found to be protective against LMP (114), and a role for LAMP in cytoprotective autophagy has been proposed (115). While overall LAMP expression is reportedly increased in a number of cancers (27, 116–118), it is likely these observations are indicative of an increase in total lysosome content and not changes in their activity per se. Given the role of LAMPs in the formation of the protective glycocalyx, it is conceivable that their overall loss augments internal hydrolase-mediated damage to other lysosomal membrane constituents and propensity toward LMP.

Lysosomes are bound by a single bilayer membrane, which is comprised of a primary lipid matrix of glycerophospholipids, sphingolipids, and cholesterol (110). In general, glycerophospholipids such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol dictate the fluidity of biomembranes and participate in trafficking, fission, and fusion events (110, 119). Lysosomal and late endosomal membranes are uniquely enriched in the glycerophospholipid bis(monoacylglycero) phosphate (BMP), permitting a heightened capacity for cholesterol transport and sphingolipid degradation (108, 120, 121). Along these lines, depletion of cellular cholesterol results in an increase in lysosomal density and affects resistance to agents such as sucrose and lytic compounds known to perturb membrane structure (122). Moreover, cholesterol contributes to the formation of detergent-resistant lipid rafts within lysosomal membranes, which are focal centers for sorting and concentrating complexes of proteins vital for trafficking and signal transduction (123) as shown by proteomic (18) and biochemical (124) analyses. Cellular repressor of E1A-stimulated genes (CREG), a secreted glycoprotein that promotes the differentiation of pluripotent stem cells (125) and inhibits cell growth (126), concentrates specifically at lipid rafts (18). Lipid rafts have garnered particular interest in recent years because of their role in metastasis and various cell death pathways (127).

Although originally considered a source of structural support, mounting evidence implicates sphingolipids like sphingomyelin, ceramide, and glycosphingolipids as important agents of lipid raft cell signaling cascades (110, 128, 129). Different sphingolipid species have been implicated in regulating cell survival, angiogenesis, inflammation, proliferation, autophagy, and programmed cell death (130–133). For example, ceramide, which is hydrolyzed from sphingomyelin by lysosomal acid sphingomyelinase (aSMase) (134) or other mechanisms (135, 136), has been intensively studied following observations that aSMase-deficient mice

were resistant to cell death induction (137, 138). The aSMase/ceramide pathway has since been identified as a central component of cellular response to various stressors and chemotherapeutics (111, 135, 139–141), potentiation of redox signaling (142, 143), autophagy (144–148) and regulation of proteins involved in programmed cell death (i.e., phospholipase A2 (149), cathepsin D (150, 151), Jun-N-terminal kinases (152).

LYSOSOMES IN CELL DEATH

Cell death is classically defined by morphological criteria: apoptotic cells display cellular shrinkage, nuclear fragmentation, and condensation into apoptotic bodies for clearance by phagocytosis; autophagy-dependent cell death involves cytoplasmic vacuolization and autophagosome formation, followed by lysosomal degradation; necrosis manifests as organelle swelling, plasma membrane breakdown, and disintegration of cellular structures (153). However, investigations into the precise biochemical and functional underpinnings of cell death processes have revealed distinct "regulated" cytotoxic programs and prompted a diversification of nomenclature. Lysosome-dependent cell death (LDCD), characterized by lysosomal destabilization and requiring LMP, is now distinguished as a subclass of programmed cell death (153). Though lysosomal rupture has been observed as an ultimate consequence of canonical cell death processes (154), primary LMP activates a death program in LDCD and can be differentiated by novel assay systems (155, 156). LMP does not generate defining morphological alterations (157) and is therefore classified at the molecular level by release of lysosomal luminal contents including proteolytic cathepsin enzymes to the cytosol, where cathepsins function in a variety of contexts as cell death executioners (158). However, the precise mechanisms leading to loss of lysosomal membrane integrity and protease translocation to the cytosol are not fully elucidated for the majority of LMP stimulants. Activities of pore-forming toxins such as venoms, bacterial toxins, and viral entry proteins are fairly straightforward; these compounds can disrupt membrane dynamics from within the lysosome following their uptake into the endolysosomal system and activation at low-pH, or alternatively induce pore formation from the cytosol (158, 159). Lysosomotropic detergents are well-studied LMP-promoting agents that function by directly disrupting membrane dynamics leading to organelle leakage or by impairing function of lysosomal lipases (158). Under physiological conditions, LDCD contributes to tissue remodeling during mammary gland involution (160) and regulates immune cell clearance following inflammation (161) or bacterial infection (162). Moreover, LDCD is associated with a variety of pathological states (153).

Consequences of LMP

LMP may either initiate or amplify a cell death cascade, and it can trigger distinct pathways depending on cellular context and the nature of lysosomal injury. The molecular players and morphological outcomes of a given lethal subroutine featuring LMP can be classified as apoptotic or necrotic, and it is widely accepted that the degree of lysosomal rupture—with respect to number of lysosomes

impacted and extent of membrane damage—dictates the specificity of lysosomal component release and downstream cellular responses (158). Extensive LMP allows rapid release of lysosome contents to the cytosol and lethal cytoplasmic acidification, resulting in rampant hydrolysis of cytoplasmic contents and cell death by necrosis following plasma membrane breakdown. Conversely, the cytosolic translocation of select cathepsins with limited LMP initiates a regulated signaling cascade and death resembling apoptosis (163–165). Indeed, cathepsin inhibition can revert the effects of limited LMP. Cathepsins were identified as principal mediators of LMP-dependent cell death by studies demonstrating cell viability rescue with pharmacological or genetic manipulation of cathepsins and their endogenous inhibitors (158, 166). Moreover, partial LMP may trigger a cytoprotective lysophagy response and cell survival if the degree of damage is sufficiently limited (167, 168). Lysosomal stress sensors activate endolysosomal damage-response mechanisms (163) whereby injured lysosomes are eliminated and recycled before the cell is committed to die. A greater understanding of the precise lysosomal membrane alterations leading to LMP is required to elucidate consequent cell fate determinations.

LMP is most widely studied in relation to caspase-mediated apoptosis-like death. Select cathepsins that remain functional at neutral pH, including cathepsins B, D, and L, can activate apoptotic effectors following limited release from leaky lysosomes (166). Indeed, cathepsins are implicated in apoptotic cancer cell death in a variety of tumor models (169). Apoptotic pathways triggered by intrinsic factors such as DNA damage, endoplasmic reticulum stress, and LMP ultimately converge on mitochondrial membrane permeabilization (MOMP) and subsequent release of pro-apoptotic factors to the cytosol (166, 170–172). Mechanistic understanding of primary LMP in apoptosis was developed largely from studies using the lysosomotropic agent Leu-Leu-methyl ester (LLOMe) (173) and other apoptotic stimuli (153). Following their cytosolic release, cathepsins can cleave Bid to generate a pro-apoptotic t-Bid fragment, thereby initiating the common intrinsic apoptosis pathway that includes t-Bid activation of pore-forming Bax and Bak proteins, MOMP, mitochondrial cytochrome c release, and activation of executioner caspases (163, 173). In fact, cathepsin inhibition reduced Bid processing and alleviated LMP, rescuing cancer cell viability (174). Cathepsins can play a variety of other roles in LMP-mediated apoptotic death involving MOMP. Cathepsins amplify signaling upstream of MOMP via proteolytic Bax activation (165) or inactivation of anti-apoptotic Bcl-2 proteins (175), and they have been shown to cleave caspases directly (158) or degrade the caspase inhibitor XIAP (176). Of note, Bax may directly permeabilize the lysosomal membrane to initiate primary LMP (153, 177, 178). LMP has also been observed downstream of MOMP as a consequence of apoptotic signaling pathways (179, 180). Reactive oxygen species (ROS) production generated by MOMP induces lysosomal membrane lipid peroxidation and LMP to perpetuate apoptotic cell death (176), while various caspases themselves play causal roles in secondary LMP (158). Under certain conditions, cathepsins regulate apoptotic death independent of caspases, such as by direct cleavage of apoptosis inducing factor (AIF) (181) or in cells with defective apoptotic machinery (182).

LMP-mediated cell death can alternatively take the form of necrosis in the absence of caspase activation, whereby cathepsins serve as the principal cell death executioner proteases (63). The caspase dependence of LDCD pathways may shift

depending on the cellular context or severity of cellular insult, as some lysosomotropic agents induce either apoptotic or necrotic cell death in a dose-dependent manner or in various cell types (173). Increased levels of oxidative stress or ATP depletion have been reported to promote a necrotic LDCD phenotype in several studies (166, 171). Necrosis, long considered an accidental and irreparable consequence of extreme chemical or physical cell stress, is now understood to be a highly regulated process with defined molecular drivers (183). Though cathepsin substrates in non-apoptotic death are not well characterized (63, 158), it is proposed that extensive LMP unleashes widespread cathepsin proteolysis and rapid breakdown of cellular structures, as cathepsin inhibition can mitigate necrotic LDCD (184–187). LMP may be an early and activating event in response to lysosome disruptors such as H_2O_2 (154), though it is also observed as a late-stage consequence of signaling in receptor interacting protein (RIP) kinase-dependent necroptosis (154). Furthermore, lysosomal ROS generation has been implicated in the execution of ferroptosis, a form of regulated cell death involving iron-dependent ROS accumulation which displays necrotic morphology (153, 188). Lysosomes degrade iron-containing proteins including ferritin during autophagy and serve as major storage sites of chelatable iron within the cell. Overloaded iron can catalyze Fenton reactions in redox cycling to produce ROS, which damage lysosomal membranes and increase the cell's susceptibility to LMP (164).

Lysosomal disruption is critical to activation of the nod-like receptor (NLR)-dependent 'inflammasome' in pyroptosis, an inflammatory cell death pathway observed in macrophages that culminates in cellular swelling, plasma membrane rupture, and cytokine release. Pyroptosis is characterized by recruitment and activation of pro-inflammatory caspase-1 by the multimodular inflammasome platform and is mediated by gasdermins, which form plasma membrane pores to drive lytic death (189, 190). Various crystals and chemical compounds induce LMP-mediated pyroptosis, and LMP is reportedly critical to NLRP3 inflammasome function, though the precise molecular pathway linking LMP and lysosomal content release with inflammasome activation is debated (191, 192). Inflammasome activation was shown to potentiate tumor invasion and stimulate angiogenesis in cases where suppressive immune cells were favorably recruited to the tumor site, such as in the absence of IL-12 (193). The pro- and anti-tumor functions of inflammasomes may thus be context-dependent, reflected by responses of NLRP3 that differ significantly depending on cell lineage (e.g., hematopoietic vs. structural epithelium) or phenotype (193).

Lysosomes serve a principal function in autophagy, an adaptive cellular stress response that is normally cytoprotective but contributes to cell death in many pathophysiological conditions including cancer (153). During autophagy, the cell digests and recycles macromolecules and whole organelles by forming double membrane-bound autophagosomes that deliver engulfed material to lysosomes (194). Considering the requirement for functional lysosomes in autophagy execution, it is perhaps unsurprising that lysosomal damage can prevent autophagosome fusion and dysregulate autophagic flux, precipitating cell death (195, 196). Cell death is commonly the consequence of experimental or pharmacological autophagy blockade, and targeting autophagic processes has emerged as a promising therapeutic strategy for treatment of diseases including cancer (197, 198). For example, lysosome-disrupting chloroquine derivatives kill tumor cells by inhibiting autophagosome-lysosome fusion and chemotherapy-induced autophagy and demonstrate

promise in clinical trials (199–201), while an aborted autophagic response following co-administration of lovastatin and farnesyl transferase inhibitor leads to non-apoptotic tumor cell death such that protein prenylation may be required for complete autophagy (202). Similarly, LMP may occur after inhibition of autophagic flux by a sophoridine analog, leading to apoptosis in pancreatic cancer cells (203). However, autophagy regulation is complex, and plays roles in cell survival, cell death, and other cytotoxic processes in a number of developmental and disease contexts (153, 204). In fact, LMP and cysteine cathepsin activity have been implicated in autophagy regulation and facilitate autophagy-mediated apoptosis in cancer cells (205–207). Indeed, numerous cell death mechanisms involve lysosome dysfunction, but the molecular interactions underlying phenotypic consequences of LMP remain largely uncharacterized.

LYSOSOMES IN CANCER

As cancer is broadly characterized by rapid cell proliferation and upregulated cell survival mechanisms to combat cellular damage, many transformation-associated changes at the level of the lysosome serve to protect the cell from LMP and LDCD. Paradoxically, the opposite effect is also observed, where neoplastic cells can sacrifice lysosomal stability in order to increase their tumorigenic potential or aggressiveness (24). Therefore, it is critical to understand the complex regulation of LMP in cancer cells in order to pharmacologically hedge the balance towards LDCD for therapeutic benefit.

Cancer cells rely on increased metabolism to sustain their rapid proliferation. The lysosome serves as a key regulator in this process and helps satisfy the catabolic need for building blocks for growth and neoplastic anabolic drive. The outer lysosomal membrane serves as a docking site for mTORC1, a signaling complex regulated by available nutrients in the cytoplasm. mTORC1 signals the upregulation of lipid and protein biosynthesis, as well as the transcription of pro-tumorigenic and anti-apoptotic regulators of the cell cycle (102, 208). Loss of several tumor suppressors including p53, PTEN, NF1 and TSC1/2 have been shown to activate mTORC1 (209). Furthermore, the lysosome is responsible for executing autophagy, one of the cell's primary mechanisms of catabolism (210). Several cancers have engaged mechanisms to constitutively activate autophagy, such as in Ras-driven pancreatic cancers. This increased baseline level of autophagic flux allows for more rapid clearance of toxic metabolites that build up as a consequence of increased metabolism (211–213). Therefore, inhibition of autophagy could potentiate induction of LMP in such tumors.

The high metabolic activity of cancer cells presents another weakness in lysosomal regulation. Swift protein turnover demanded by rapidly dividing cancer cells leads to excessive intralysosomal accumulation of iron (214). Iron accretion alone is sufficient to sensitize the lysosome to LMP; however, Fenton-type reactions with H_2O_2 can generate additional ROS and further destabilize lysosomal membranes through lipid oxidation. Coupled with the well-documented increase in ROS production in cancer cells and augmented cytoplasmic levels of cathepsins, lysosomal iron accumulation sensitizes tumor cells to LMP and LDCD (215). Other transformation-associated alterations in cancer cell lysosomes include

increases in expression of lysosomal enzymes, changes in lysosomal morphology and localization, and modifications in lysosome-associated proteins. Cathepsins are highly expressed in cancer cells and are localized to the cell periphery in secretory lysosomes, where they can be secreted into the surrounding extracellular environment through a mechanism similar to the lysosomal exocytosis that allows the formation of invasive protrusions in *C. elegans* development (216). Once in the extracellular space, they wreak havoc by cleaving a variety of adhesion proteins, degrading the basement membrane, and releasing sequestered growth factors such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), leading to neoplastic progression through invasion, angiogenesis, and metastasis (41, 217, 218). Additionally, downregulation of a potent regulator of pericellular cathepsin accumulation, M6PR, in a rat cell model of hepatocellular carcinoma abrogates inhibition of pro-neoplastic cathepsin activity (219). High concentrations of cathepsins predict increased tumor aggressiveness and poor prognosis in many tumor types such as breast cancer, lung and colorectal carcinomas, and gliomas (220–223). However, increased levels of cytosolic cathepsins also sensitize the cell to LDCD (224). Increased lysosomal cathepsin activity leads to decreased LAMP-1 and -2 levels in ERK-, ErbB2-, and K-Ras-driven models of cancer, reducing lysosomal membrane stability and rendering the organelle susceptible to LMP (114). These cancer-associated vulnerabilities illuminate a potential therapeutic window to selectively target tumor cells.

As previously noted, lysosomes are alternatively localized within transformed cells; the switch from a perinuclear position to the plasma membrane facilitates secretion of toxic contents into the extracellular space, promoting extracellular acidification and activation of secreted lysosomal hydrolases (41). Increased expression of v-ATPase in metastatic tumor cells also contributes to extracellular acidification (225). Additionally, cancer cells exhibit increased lysosomal size, a phenomenon correlated with the metastatic potential of breast cancer cells (28). Once again, these tumorigenic changes compromise lysosomal stability, sensitizing tumor cells to LMP (28). Problematically, some forms of cancer have developed mechanisms to overcome this increased sensitivity to LMP. Breast cancer cells show elevated expression of Hsp70 (226), which has been shown to rescue lysosomal membrane integrity by stabilizing intralysosomal aSMase interaction with the critical lipase cofactor BMP (30, 215). Likewise, mammary-derived growth inhibitor (MDGI), which contributes to maintenance of lysosomal integrity, is a marker of invasiveness in human glioblastoma patient-derived cells that are resistant to chemoradiation. Silencing MDGI leads to alterations in lysosomal membrane lipid composition through reduced trafficking of polyunsaturated fatty acids into the lysosomes, leading to eventual LMP-dependent cell death (227). Along these lines, targeting Hsp70 or other molecules critical to lysosome stability and function could prove to be promising therapeutic strategies.

Localization of transport proteins to the lysosomal membrane in transformation confers therapeutic resistance via active sequestration and inactivation of antineoplastics within the lysosomal lumen. Canonically, members of the ATP-binding cassette (ABC) family of transporters reside in the plasma membrane where they expel cytoplasmic antineoplastics, but some cancers (e.g., leukemia, breast and cervical cancers) exhibit lysosomal expression of transporters like P-glycoprotein (P-gp or ABC1), leading to drug sequestration in the lysosome (228–230). Inhibition of P-gp in cancer cells restores sensitivity to the sequestered drugs and

hyper-sensitizes cells to chemotherapeutic death (228, 230). Overexpression of ABCA3 correlates with poor prognosis in acute myeloid leukemia patients, and ABCA3 localizes to lysosomes in a chronic myeloid leukemia cell line (231, 232). The transport protein ATP7B, a copper transporter, is overexpressed in many cancers (233) and serves to sequester and exocytose platinum-based antineoplastics (234, 235). Overall, the dynamic changes in cancer-associated lysosomes reveals a wide range of possible therapeutic options for exploration (236).

MODULATION OF LMP AND ITS THERAPEUTIC POTENTIAL

Depletion of cancer cells via LMP is an attractive therapeutic strategy, holding particular promise for combating apoptosis-resistant cancer cell populations (237). Initial interest in this regard was sparked by realization of the degradative potential of lysosomes (1), catalyzing the search for pharmacologic agents that would destabilize the lysosomal membrane to kill cancer cells from the inside. Cholesterol and hydrocortisone were identified as stabilizing agents (32), while weakly basic amines with long hydrophobic tails disturbed membrane structure and induced LMP (238–240). However, enthusiasm quickly waned upon recognition that lysosomes were a ubiquitous feature of nearly all cells (excluding erythrocytes (34)), and would not permit the distinction between normal and cancer cells (32). Interest has since reignited following more recent studies that suggest cancer-associated lysosomes express unique features (i.e., size, hydrolase content, membrane fragility) that may make them suitable for targeting. Numerous lysosome-disrupting agents are currently under investigation or in clinical development for cancer and other indications (237, 241), though clinical data demonstrating efficacy of these approaches is limited. This section outlines select agents known to induce LMP, with a particular emphasis on their potential as anticancer therapeutics. General mechanisms of LMP induction are illustrated in Figure 2.

Lysosomotropic Agents

Weakly basic amine compounds rapidly accumulate within lysosomal lumens and are thus referred to as 'lysosomotropic' (242). Sequestration of amine-containing agents occurs by a non-enzymatic and non-transporter mediated cation-trapping mechanism, referred to as 'lysosomal trapping' (44, 243, 244), but may also occur by endocytosis or facilitated transport (245). In particular, CADs and lysosomotropic detergents feature a hydrophobic ring structure and a hydrophilic side chain with a charged cationic amine group that allow them to readily diffuse across cellular membranes in their non-ionized state. However, exposure to the acidic interior of the lysosome leads to protonation and entrapment (242). As these agents accumulate within the lysosomal lumen, they interact with negatively charged intra-lysosomal vesicles, displacing associated enzymes and lipid binding proteins and inducing swelling and vacuolization due to an influx of water into the lysosomal lumen (242). The appearance of lamellar bodies that occurs in response to some CADs (43) signals the accumulation of lipids within the lysosome that may occur following the interaction of drugs with phospholipids (246)

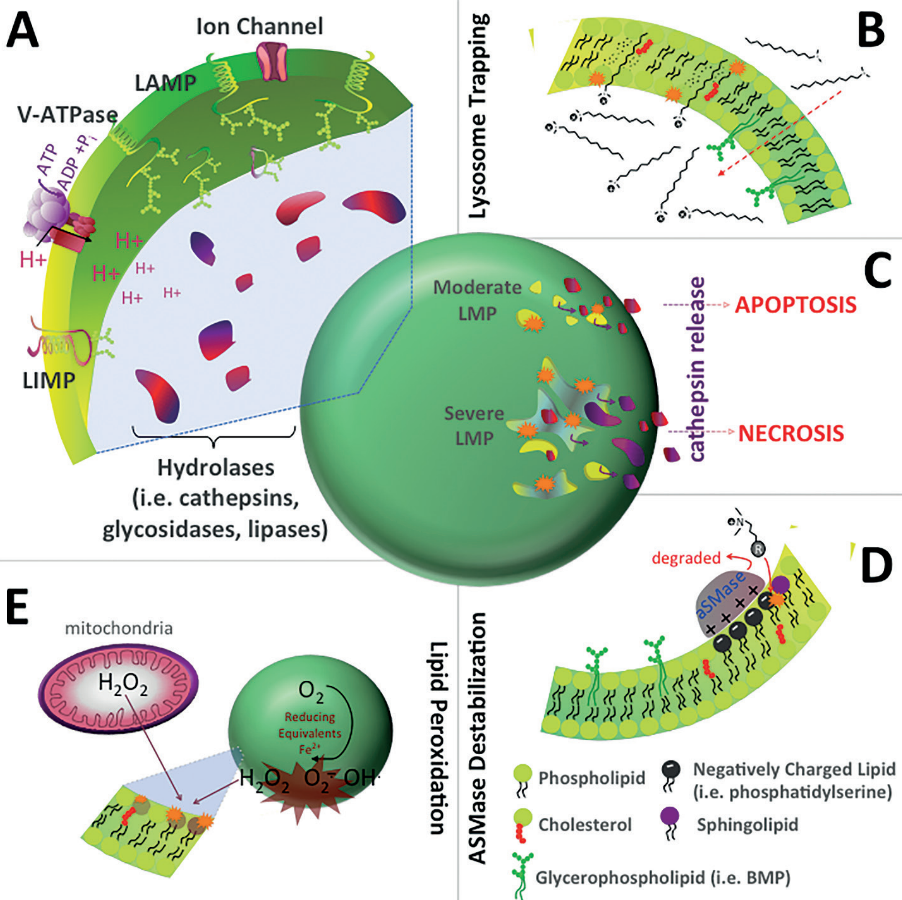

Figure 2. Mechanisms of LMP induction. (A) The lysosomal membrane is expanded to show detail. Carbohydrates on lysosomal associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs) form the protective glycocalyx. Also, resident within lysosomal membranes are ion channels and the vacuolar ATPase (v-ATPase) that maintains the acidic interior of the lysosome. A number of hydrolases are found within the lysosomal lumen. Dysregulation of these endogenous factors can contribute to LMP. **(B)** LMP-inducing agents including many lysosomotropic detergents and cationic amphiphilic drugs (CADs) contain hydrophilic side chains with charged cationic amine groups, allowing them to passively partition across cellular membranes in their non-ionized state. Within the acidic lysosomal lumen, these agents become protonated and sequestered in a process termed 'lysosomal trapping.' **(C)** The degree of LMP often dictates the ensuing course of cell death (apoptosis or necrosis). **(D)** Expansion of the lysosomal membrane to show details of lipid constituents: the lysosomal membrane is composed of phospholipids, glycerophospholipids such as bis(monoacylglycero)phosphate (BMP), cholesterol, and sphingolipids such as sphingomyelin and ceramide. Sphingomyelin is hydrolyzed to ceramide by acid sphingomyelinase (aSMase). Positively charged amino acids on aSMase allow it to interact with negatively charged head groups on lipids such as phosphatidylcholine or phosphatidylserine. aSMase is displaced by agents that interfere with this binding domain. **(E)** Mitochondrial reactive oxygen species (ROS) production stimulates lipid peroxidation and LMP. Lysosomal membrane peroxidation is augmented by production of reactive hydroxyl radicals generated through degradation of iron-containing molecules within the lysosomal lumen in the presence of reducing agents.

or via inhibition of phospholipid metabolism (247, 248). The anti-tumor efficacy of CADs, including FDA-approved anti-histamines (227), anti-depressants (249), and anti-malarials (250), has been documented in both experimental and observational studies. The CAD 5-(N,N-hexamethylene) amiloride (HMA) showed robust induction of necrotic cell death in breast cancer cells regardless of molecular profile, with little toxicity against untransformed cells (184). Another CAD, the anti-histamine clemastine, killed patient-derived glioblastoma cells by LMP but was minimally toxic in normal human astrocytes and murine brain endothelial cells, pointing to a critical therapeutic window for the treatment of this highly aggressive and chemo-refractory disease (227). Lysosomotropic detergents, which combine weakly basic amines (e.g., imidazole, morpholine) with long (9–14 carbons) and straight hydrocarbon tails, are characterized by membrane disruptive surfactant properties that progress with continued lysosomal accumulation (238–240). Derivatives of imidazole and morpholine lysosomotropic detergents were originally developed as anticancer therapeutics, inducing apoptosis or necrosis with LMP in a dose-dependent fashion across a range of cancer cell types (239, 251–253).

Interestingly, basic amines are a ubiquitous feature of therapeutic agents, conferring varying degrees of lysosomotropic potential (254). As such, lysosomal drug sequestration may either be cytotoxic or cytoprotective depending upon whether it potentiates LMP or prohibits interactions with the intended target. Indeed, substantial evidence supports the notion that the lysosome contributes to chemotherapeutic resistance (255–258). In one such mechanism, cellular stress resulting from accumulation of lysosomotropic drugs was shown to trigger exocytosis, leading to lysosome-mediated multidrug resistance (259). Furthermore, the degree of resistance to the topoisomerase II inhibitor C-1330 and the receptor tyrosine kinase inhibitor sunitinib was directly associated with the total number of lysosomes (258) and, perhaps more importantly, the degree to which the normal cytosol-to-lysosome pH gradient is altered within a given cancer cell (260). Similar to other anti-cancer therapeutics (doxorubicin, mitoxantrone), C-1330 and sunitinib were shown to preferentially accumulate within lysosomes, triggering substantial, dose-dependent increases in lysosome number, size, and their ability to uptake the lysosomal marker LysoTracker Red (258). Poor lysosomal accumulation and retained drug sensitivity was associated with intrinsic disruption of the physiologic pH of some cancer cell lysosomes (i.e., MCF7 breast cancer cells) (260). Accordingly, diminished lysosomal entrapment of weakly basic amines could be replicated with pharmacological disruption of the pH gradient following v-ATPase inhibition by bafilomycin A (255, 260) or administration of the lysosomotropic agent chloroquine (255), suggesting that modulation of lysosomal pH may be an effective strategy to overcome chemoresistance.

Indeed, the pH-disrupting agents chloroquine and hydroxychloroquine have been investigated as anti-cancer therapeutics, with dozens of clinical trials in progress (241, 261–265). Long used as an antimalarial, chloroquine sensitizes cancer cells to radiation and chemotherapy. Although thought to convey therapeutic sensitivity through the inhibition of protective autophagy, recent evidence suggests its role may be more complicated (266–268), and may at least partially involve the capacity to overcome drug sequestration within lysosomes (255, 269, 270). Nevertheless, as a nonspecific inhibitor of autophagy, potential side effects may arise from a loss of protective autophagy within normal tissues

(i.e., brain, liver, heart, kidney) that occurs during therapeutic intervention (271). Along these lines, chloroquine-treated mice are more likely to suffer from kidney damage in a model of ischemic–reperfusion than untreated animals (272). Chloroquine uptake may also be reduced in the external acidic milieu of some tumors, reducing its efficacy under these conditions (273). However, particular derivatives of chloroquine (273) or other small molecule inhibitors of autophagy (264) may offer improved stability and potency and warrant further study.

Nanoparticles

While nanoparticles have been extensively investigated for efficient tumor-site delivery of anti-cancer drugs in recent years, issues of non-specific cell toxicity are often attributed to lysosome dysfunction, as nanoparticles can accumulate in lysosomes and induce LDCD themselves. However, the precise mechanisms underlying nanoparticle toxicity are debated, with the involvement of autophagy in question in various cancers (274, 275). Several studies reported that early induction of autophagy allowed rapid nanoparticle uptake and delivery to lysosomes—a requirement for zinc oxide nanoparticle (ZnONP)-mediated cytotoxicity, i.e., nanoparticle dissolution and content release within the lysosomal lumen (276)—but that subsequent lysosomal damage resulting from nanoparticle buildup impaired autophagic flux and ultimately resulted in cell death (276, 277). Moreover, ZnONP cytotoxicity was not attributed to nanoparticle dissolution and zinc ion release within lysosomes in cellular models of leukemia and normal red blood cells but rather to LMP triggered by intact nanoparticles (278). A variety of nanoparticle formulations are currently under investigation as novel lysosome-disrupting cancer therapeutics (279, 280). Interestingly, a recent report demonstrated that nanoparticles carrying small interfering RNA (siRNA) therapeutics can become sequestered in lysosomes and exhibit inefficient endolysosomal escape (281), while CAD administration promotes LMP and cytosolic siRNA delivery.

Sphingolipids

Mounting evidence suggests that LMP occurs following specific changes in the composition of membrane lipids and major lysosomal proteins (114). Particularly interesting data has recently come to light suggesting that cancer cells have perturbed lipid species as compared to their normal counterparts, a feature that may permit their selective depletion. Along these lines, the lysosomotropic detergent siramesine and similar compounds directly induce LMP and non-apoptotic cell death in transformed cells but not in oncogene-depleted ('detransformed') or non-transformed variants (29, 282). These effects were universal across all cancer types tested (breast, ovary, prostate, cervix and bone (29)). Intriguingly, similar cancer specific effects were not found for other compounds that also induce LMP (i.e., LLOMe, sphingosine) or neutralize pH (concanamycin A) (29), although the lack of specificity may be related to the dose used (283).

Ostensibly, siramesine cytotoxicity is mediated by the displacement of aSMase (29), normally responsible for the breakdown of sphingomyelin to ceramide at the inner lysosomal membrane (134). Augmentation of aSMase activity occurs in

stressed normal cells following induction of heat shock protein 70 (Hsp70), which then binds the glycerophospholipid BMP to activate aSMase at the lysosomal membrane (30). The constitutive elevation of Hsp70 has been detailed for a number of cancer types, and is associated with resistance to caspase-dependent and -independent cell death and poor prognosis (284–287). Bolstered aSMase activity appears to contribute to lysosomal integrity (29), making it a particularly attractive therapeutic target. Accordingly, exposure to siramesine (29) or Hsp70 small molecule inhibitors (288) results in reductions in aSMase activity, induction of LMP, caspase-independent cell death and enhanced sensitivity to chemotherapeutics. In agreement with siramesine-mediated aSMase inhibition, depleted Hsp70 induces effects that are cancer cell specific (286, 289–292). It is likely that aSMase inhibition perturbs lipid ratios (293), leading to membrane fragility and a propensity for LMP (29). This postulate is further supported by studies suggesting that augmented sphingomyelin content alone destabilizes lysosomal membranes (30), is selectively toxic to transformed cells (294), inhibits autophagic flux (295), and impairs intracellular vesicle and plasma membrane fusion events (296). Destabilized lipid content following aSMase inhibition also affects signaling events at the plasma membrane, including the clustering and signaling of K-Ras (293), a protein with a dominant role in cell proliferation and survival. These data suggest that aSMase inhibition may not only be cancer cell specific (29), but may also be particularly well suited for K-Ras-driven cancers (i.e., pancreas, colon and lung), which currently lack targeted therapeutic options (297).

The clinical significance of sphingolipid species is further substantiated by observations that perturbed ceramide clearance was directly correlated with reduced chemotherapeutic sensitivity (298). As such, modification of ceramide levels by enhancing *de novo* biosynthesis or modulating aSMase activity have also been suggested as potential anti-cancer strategies to overcome imbalances in lysosomal ceramide (298–301). Although a clear link has yet to be established, a recent study demonstrated that markedly enhanced aSMase potentiates the accumulation of ceramide and triggers cathepsin B release via LMP upstream of apoptosis (176). Importantly, cathepsin B catalyzes the degradation of XIAP (X-linked inhibitor of apoptosis) (176), the upregulation of which has been associated with therapeutic resistance and poor survival (302–304).

It is worthwhile to note that experimental modulation of aSMase activity appears to produce seemingly contradictory results. On the one hand, the depletion of aSMase results in sphingolipid accumulation, ceramide depletion, LMP, and non-apoptotic cell death (29), whereas on the other hand its overexpression precipitates ceramide accumulation, LMP, and apoptosis (176). As such, it is likely that lysosome integrity depends upon the precise balance of sphingolipid species. Moreover, it is conceivable that there are factors modified under conditions of aSMase depletion or overexpression that await further investigation. For example, sphingosine accumulation inhibits cholesterol export (305), which may have implications for signaling events at lysosome-associated lipid raft domains or in membrane dynamics. Moreover, modifications to sphingosine or ceramide may generate either sphingosine-1-phosphate or sphingosine, respectively. Sphingosine-1-phosphate binds to G-coupled protein receptors to regulate growth, survival and migration of cells, and is associated with malignant transformation (306). Sphingosine was shown to induce LMP and programmed cell death (307–309), albeit nonspecifically (29). It was proposed that accumulated

sphingosine permeabilizes the membrane in a detergent-like fashion, resulting in cell death (310). A potential mechanism is outlined in Figure 3 that discusses the intricate balance of sphingolipids in LMP induction.

Calcium

Interestingly, sphingosine was also shown to affect calcium release from the lysosomal membrane (311). Although not often considered a major calcium storage site, the reported calcium concentration of 500μM within the lysosomal lumen (312) is comparable with that of the endoplasmic reticulum (313). In studies addressing the pathology of Niemann-Pick disease type C (NPC), a rare progressive disorder characterized by the accumulation of sphingolipid species and cholesterol, sphingosine was shown to induce the specific release of calcium from lysosomes and late endosomes but not release from other intracellular storage

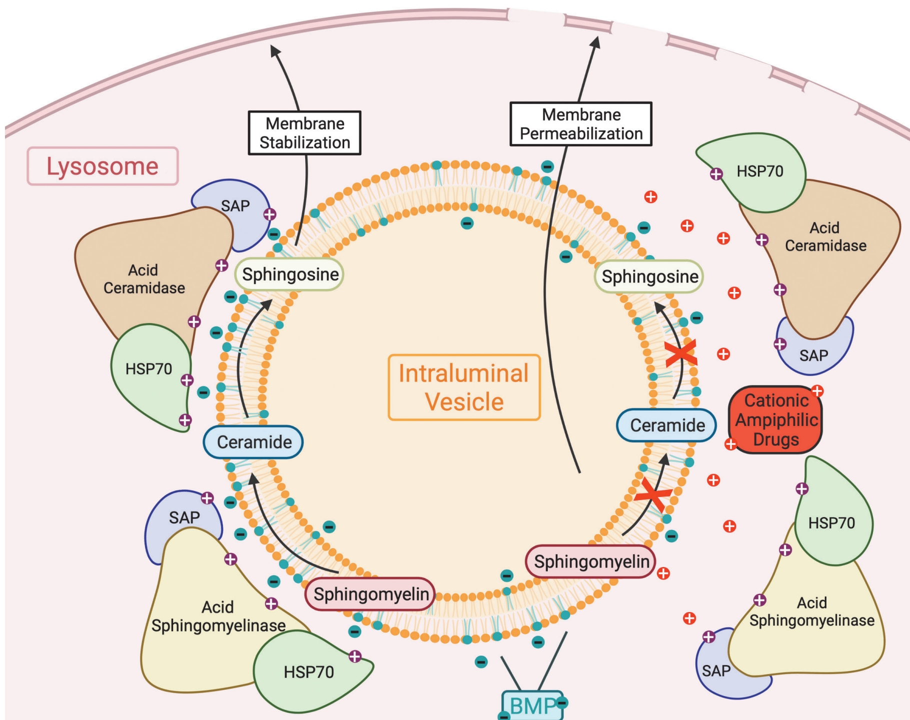

Figure 3. Membrane stabilization and permeabilization is dependent on the balance of sphingolipids in lysosomes. Negatively charged bis(monoacylglycero)phosphate (BMP; shown in teal) is enriched in membranes of intraluminal lysosomal vesicles and provides docking points for positively charged acid sphingomyelinase, acid ceramidase, and other co-factors such as heat shock protein 70 (HSP70) and saposins (SAP). Proper recruitment of these enzymes facilitates lipid metabolism and membrane stabilization. Cationic amphiphilic drugs (CADs) disrupt BMP-enzyme interactions, inhibiting lipid metabolism and resulting in lysosomal membrane permeabilization. Figure created with BioRender.com.

sites (311). This rise in cytosolic calcium was dependent on interaction of sphingosine with lysosome and endosome calcium channels (two-pore channel 1) (311). Lysosome-released calcium is vital for vesicle fusion and secretion, autophagy, and lysosomal biogenesis (39, 314–316), as well as apoptosis-dependent phosphatidylserine externalization (317), an early recognition signal for engulfment by phagocytic cells (318, 319). Intriguingly, prolonged endoplasmic reticulum stress results in pancreatic cancer cell death mediated by LMP and accumulation of cytosolic calcium (320). General increases in intracellular calcium are also associated with the activation of cytosolic calpain proteases and the initiation of LMP. Along these lines, μ-calpain is capable of permeabilizing the membrane of isolated lysosomes (321). Further, activated calpain species were shown to localize to the lysosomal membrane prior to cathepsin release (186, 322, 323), whereas their pharmacological inhibition effectively abolishes LMP (324).

Reactive oxygen species

Increased production of ROS (i.e., singlet oxygen and hydrogen peroxide) stimulates lipid peroxidation and destabilization of the lysosomal membrane (325–327). Cell death depends on the degree of LMP, where minimal leakage of cathepsins is nonlethal, reversible (328), and primarily impacts cell proliferation, while moderate or high membrane destabilization respectively induce apoptosis and necrosis (170). Peroxidation of the lysosomal membrane is likely mediated by local production of highly reactive hydroxyl radicals. Since iron-containing macromolecules are degraded within the lysosomal lumen in the presence of reducing agents (i.e., glutathione, ascorbic acid and cysteine), there is a capacity to generate reactive radicals with exposure to H_2O_2 (329). Accordingly, iron chelation is protective against LMP whereas accumulation of iron-containing proteins or iron complexes sensitizes lysosomes to membrane damage (330–336). Cancer-associated lysosomes may have a heightened propensity to accumulate iron, given the enhanced turnover of iron-containing proteins that accompanies their rapid proliferation (170). Consequently, cancer cells may be more sensitive to ROS-induced LMP (214). Iron has also been implicated in maintenance of the cancer stem cell (CSC) state, and salinomycin selectively kills cancer stem cells by sequestering iron in lysosomes, leading to ROS-mediated LMP and ferroptotic cell death (337). Unsurprisingly, aSMase was implicated in ferroptosis initiation through a positive feedback loop of ROS production, further connecting key lysosomal enzymes to ferroptosis (338).

While iron-loading has not been vetted as an anti-cancer strategy, such an approach may offer particular benefit to hypoxic regions of tumors where limited ROS production might contribute to insensitivity to LMP (329). Iron loading of hypoxic cells may sensitize them to ionizing radiation or other ROS-generating agents. On the other hand, redox-active iron has been suggested as a mutagen, contributing to persistent oxidative stress of DNA damage (339–341). Iron-chelation also has important clinical applications, protecting normal tissues from radiation damage during cancer treatment (342). An exception is CAD iron-chelating compounds, as they remain trapped within lysosomes and lead to iron-starvation and death (343, 344).

CONCLUSION

The lysosome is clearly more than a cellular garbage disposal, contributing to dynamic processes that are essential to both normal physiologic function and disease pathology. Cancer-associated changes in lysosomal structure and function may bolster therapeutic resistance but may also be the gateway to cancer cells' ultimate demise. Indeed, emerging data suggests the lysosome is potentially a powerful anti-cancer target, given specific alterations in cancer cells that are not seen in non-transformed counterparts. Moreover, cancer cells frequently evade therapy-induced apoptosis due to intrinsic or acquired mutations in caspase-dependent pathways; thus, utilization of LDCD and other caspase-independent cell death programs will be critical in the future development of cancer therapeutics. Nevertheless, a number of questions still remain with respect to cancer-associated lysosome signaling, membrane dynamics, ion regulation, and the precise role of LMP in cell death, which have limited the number of lysosome-targeted therapeutics transitioning from preclinical study to clinical development. The dual functionality of cathepsin activation in cancer cell invasion and LDCD is a critical area requiring further investigation, as cathepsins are important mediators of both pro-tumorigenic and pro-death processes, depending on context. Substantial progress on these and other fronts will likely be fueled by continued advances in methods for the detection and quantification of lysosome-associated events.

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