

Review

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Emerging mechanisms of drug-induced phospholipidosis

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Abstract: Drug-induced phospholipidosis is a lysosomal storage disorder characterized by excessive accumulation of phospholipids. Its cellular mechanism is still not well understood, but it is known that cationic amphiphilic drugs can induce it. These drugs have a hydrophilic amine head group that can be protonated in the endolysosomal compartment. As cationic amphiphiles, they are trapped in lysosomes, where they interfere with negatively charged intralysosomal vesicles, the major platforms of cellular sphingolipid degradation. Metabolic principles observed in sphingolipid and phospholipid catabolism and inherited sphingolipidoses are of great importance for lysosomal function and physiological lipid turnover at large. Therefore, we also propose intralysosomal vesicles as major platforms for degradation of lipids and phospholipids reaching them by intracellular pathways like autophagy and endocytosis. Phospholipids are catabolized as components of vesicle surfaces by protonated, positively charged phospholipases, electrostatically attracted to the negatively charged vesicles. Model experiments suggest that progressively accumulating cationic amphiphilic drugs inserting into the vesicle membrane with their hydrophobic molecular moieties disturb and attenuate the main mechanism of lipid degradation as discussed here. By compensating the negative surface charge, cationic enzymes are released from the surface of vesicles and proteolytically degraded, triggering a progressive lipid storage and the formation of inactive lamellar bodies.

Keywords: acid sphingomyelinase; cationic amphiphilic drugs; lysosomal lipid degradation; lysosomal phospholipase; lysosomotropism; phospholipid.

Introduction

Drug-induced phospholipidosis (DIPL) is caused by long-term treatment of animals and humans with cationic amphiphilic drugs (CADs) leading to an intracellular accumulation of phospholipids in kidney, liver, lung, brain, cornea and other organs (Lüllmann et al., 1975). DIPL is characterized by the appearance of cytosolic inclusions consisting of concentric myelin-like structures, termed lamellar bodies that are of lysosomal origin (Figure 1) (Reasor and Kacew, 2001).

DIPL was first observed in rats that had been treated with the antimalarial drug chloroquine (Nelson and Fitzhugh, 1948), which is a CAD (Table 1). Lamellar bodies are also observed in some inherited lipid storage disorders and contain non-degradable sphingolipids and phospholipids (Lüllmann et al., 1975; Sandhoff and Harzer, 2013) mainly accumulating in late endosomes and lysosomes (Breiden and Sandhoff, 2019). More than 50 drugs in clinical use have been identified so far to induce phospholipidosis. They include antidepressants, antipsychotics, antibiotics, antihistaminics, antiarrhythmics and antimalarial drugs (Table 1). Most of them are CADs and have some common molecular properties. They are lysosomotropic and contain a hydrophilic amine head group that can be protonated in the acidic endolysosomal compartment to carry a positive charge (Table 1), and a hydrophobic tail consisting of an aromatic or aliphatic ring structure (Table 1) that can anchor in lipid bilayers of cellular membranes. Due to the great structural variability of the CADs known, the onset of a phospholipidosis, the possible appearance of clinical symptoms (inflammatory reactions, macrophagic infiltrations and fibrosis) varies widely with their specific molecular structures and properties (Kodavanti and Mehendale, 1990).

Besides lipids, also CADs have been observed to accumulate in lysosomes during drug treatment (Kodavanti and

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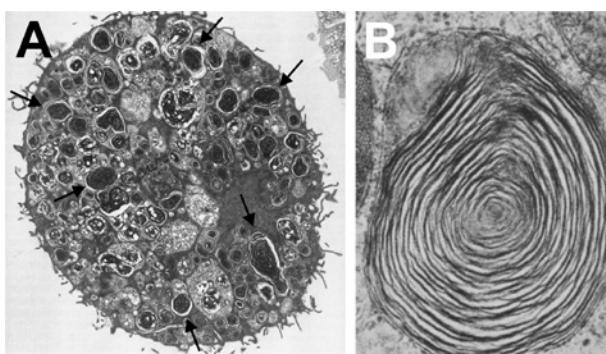


Figure 1: CADs induced lamellar bodies resemble the MCBs (membranous cytoplasmic bodies) observed in Tay-Sachs disease and other GM2 gangliosidoses (Samuels et al., 1963). (A) Alveolar macrophage from a rat treated with chlorphentermine shows numerous lamellar bodies (arrows). Picture taken from (Hook, 1991). (B) Drug-induced lamellar body of a chromaffin cell of a rat treated with 1-chloro-amitriptyline. Electron micrograph taken from Lüllmann et al. (1978).

Mehendale, 1990). Even treatment of cultured fibroblasts with low doses of CADs triggers lysosomal alterations, like the formation of lamellar storage bodies (Lüllmann et al., 1975). These bodies vary tremendously in their pattern of lipid storage and in their size (Anderson and Borlak, 2006). They appear as uni- or multi-lamellar particles that have an acidic character like lysosomes, suggesting that they are caused by an impaired lysosomal breakdown of complex lipids.

Despite a longstanding investigation of the DIPL, no unifying concept has been developed to understand the molecular and cellular mechanisms of its development. An accumulation of phospholipids could be explained by an inhibition of phospholipid degradation or an enhanced phospholipid biosynthesis. Therefore, several mechanisms have been proposed like the direct inhibition of lysosomal phospholipases by drugs or an induction of non-degradable drug-phospholipid complexes. Furthermore, an inhibition of lysosomal enzyme transport and an enhanced phospholipid and cholesterol biosynthesis have been discussed (Joshi et al., 1988; Sawada et al., 2004; Nonoyama and Fukuda, 2008; Lowe et al., 2012). Also, *in silico* studies predicted that DIPL could be induced by an inhibition of lysosomal phospholipase activity and an enhanced cholesterol biosynthesis (Lowe et al., 2012).

Studies on sphingolipid catabolism in health and in lysosomal storage diseases caused by inherited defects in degradation, however, revealed several molecular and cellular mechanisms that are basic for lysosomal function and physiological lipid turnover in general (Figure 2). Many of these essential mechanisms are strongly affected by an accumulation of CADs in the endosomal and

lysosomal compartments. Therefore, we propose the hypothesis that the impairment of these mechanisms is key for the development of an induced lipidosis.

Lysosomal lipid degradation

Lysosomes were observed by de Duve and colleagues as acidic cytoplasmic organelles (de Duve and Wattiaux, 1966). They are involved in a variety of cellular processes, especially in the catabolism of macromolecules including complex lipids, defense against pathogens, cholesterol homeostasis, lipid and energy homeostasis, apoptosis, bone remodeling, repair of the plasma membranes and cell signaling (Pu et al., 2016).

Lipids reach the lysosomal compartment mainly by autophagy and endocytotic processes. Neutral lipids (tri-glycerides, diglycerides, ceramides, cholesterol and cholesterol esters) are hydrophobic and, as free molecules, insoluble in aqueous solutions and in the cytosol, but can occur in lipophilic phases of adipocytes, in lipid droplets of many other cells and also in the hydrophobic core of biological membranes. Amphiphilic phospholipids and sphingolipids, however, occur as bilayer forming components, mainly in biological membranes. All these lipids can reach lysosomes and intralysosomal luminal vesicles (ILVs) for degradation by various processes of endocytosis (Figure 2A) and autophagy. Under normal physiological conditions, they are split into their components by hydrolases in the lysosomal compartment, releasing fatty acids, glycerol, sphingoid bases and monosaccharides, which are secreted into the cytosol of the cell and used as fuel of energy metabolism or as building blocks for the synthesis of new cellular macromolecules (Kolter and Sandhoff, 2005).

The lysosomal compartment contains more than 50 different hydrolytic enzymes, including lipases, phospholipases, glycosidases, phosphatases, sulfatases, proteases and nucleases, besides lipid binding and transfer proteins and further proteins of still unknown function (Eskelin et al., 2003; Sandhoff and Sandhoff, 2018). Most of them are soluble glycoproteins, some of them use hydrophobic domains to interact with membranes and to recognize their lipid substrates. Others need lipid binding and transfer cofactors, as many sphingolipid catabolizing hydrolases, to recognize their (glyco)sphingolipid substrates (Sandhoff and Sandhoff, 2018).

Due to their insolubility in aqueous solutions and based on experimental evidence obtained *in vitro* and in cell cultures with mutant human and murine cells, we

Table 1: Phospholipidotic pathological changes.

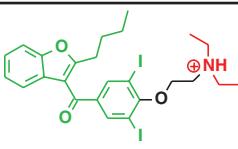
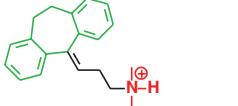
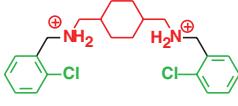
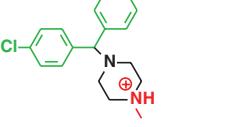
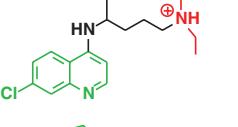
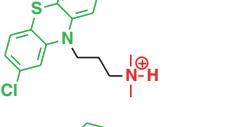
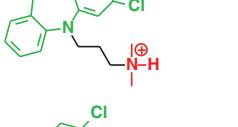
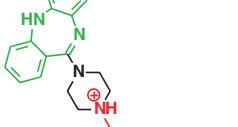
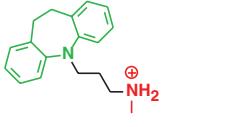
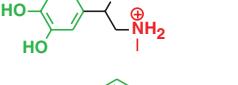
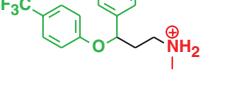
Compound	Therapeutic categories	Structure ^a	pKa ^b	clogP ^c	DIPL <i>in vivo</i> ^d	DIPL <i>in vitro</i> ^e	References	
Amiodarone	Antiarrhythmic		8.5	7.9	+	H, R	+	Riva et al. (1987), Sawada et al. (2004), Hanumegowda et al. (2010)
Amitriptyline	Antidepressant		9.8	4.9	+	R	+	Pelletier et al. (2007), Vitovič et al. (2008), Hanumegowda et al. (2010), Lowe et al. (2012)
AY-9944	Antihyperlipidemic		9.1	5.8	+	R, Ra, M	+	Yoshida et al. (1985), Sawada et al. (2004), Pelletier et al. (2007)
Chlorcyclizine	Antihistaminic		7.6	4.2	+	R	+	Kacew (1982), Sawada et al. (2004)
Chloroquine	Antimalarial		10.3	4.3	+	H, R	+	Hanumegowda et al. (2010)
Chlorpromazine	Antipsychotic		9.2	4.6	+	R	+	Sawada et al. (2004), Hanumegowda et al. (2010), Fischer et al. (2012)
Clomipramine (Chlorimipramine)	Antidepressant		9.2	4.5	+	R	+	Sawada et al. (2004), Hanumegowda et al. (2010)
Clozapine	Antipsychotic		7.6	3.4	+	R	+	Hanumegowda et al. (2010), Fischer et al. (2012)
Desipramine	Antidepressant		10.0	3.9	+	R?	+	Hanumegowda et al. (2010)
Erythromycin	Antibiotic		8.9	3.1	+	R	+	Hanumegowda et al. (2010)
Fluoxetine	Antidepressant		9.8	4.2	+	H, R	+	Sawada et al. (2004), Hanumegowda et al. (2010), Fischer et al. (2012)

Table 1: (continued)

Compound	Therapeutic categories	Structure ^a	pKa ^b	clogP ^c	DIPL <i>in vivo</i> ^d	DIPL <i>in vitro</i> ^e	References	
Gentamicin	Antibacterial		10.2	-3.1	+	H, R	Hanumegowda et al. (2010)	
Haloperidol	Antipsychotic		14.0	3.7	+	R?	+	Hanumegowda et al. (2010), Fischer et al. (2012)
Imipramine	Antidepressant		9.2	4.3	+	R	+	Hanumegowda et al. (2010), Fischer et al. (2012)
Ketoconazole	Antifungal		6.8	4.2	+	M	+	Sawada et al. (2004), Hanumegowda et al. (2010)
Perhexiline	Antianginal		10.6	5.5	+	H, R	+	Sawada et al. (2004), Hanumegowda et al. (2010)
Promazine	Antipsychotic		9.2	3.9	+	R	+	Kodavanti et al. (1990), Fischer et al. (2012)
Propanolol	Antiarrhythmic		9.7	2.6	(+)		+	Hanumegowda et al. (2010), Fischer et al. (2012)
Sertraline	Antidepressant		9.9	5.2	(+)		+	Sawada et al. (2004), Hanumegowda et al. (2010), Fischer et al. (2012)
Tamoxifen	Antiestrogenic		8.8	6.4	+	R	+	Sawada et al. (2004), Hanumegowda et al. (2010)
Tilorone	Antiviral		9.7	4.3	+		+	Fischer et al. (2012)
Triparanol	Cholesterol synthesis inhibitor		8.9	6.7	+	Ha		Arai et al. (1967)

^aRed, hydrophilic amine head group that can be protonated in the acidic endolysosomal compartment; green, hydrophobic tail consisting of an aromatic or aliphatic ring structure.

^bValues for protonation (logarithmic acid dissociation constant) (pKa) of the most basic group.

^cValues for lipophilicity (logarithmic partition coefficient) (clogP).

^dDIPL *in vivo*: H, Human; R, rat; M, mice; Ra, rabbit; Ha, hamster.

^eDIPL *in vitro* from cell based assay by phospholipidotic pathological changes.

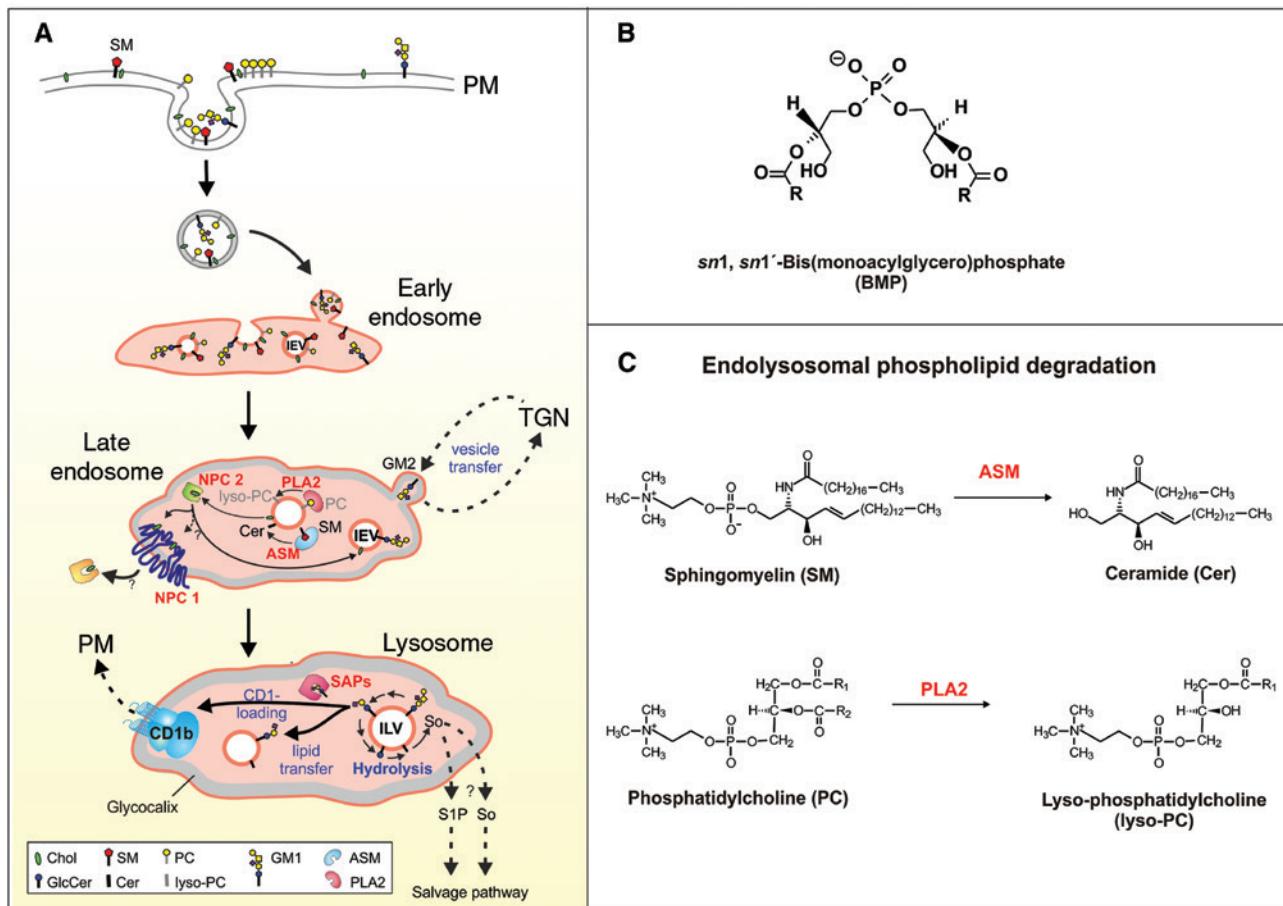


Figure 2: Schematic pathway of phospholipid and sphingolipid catabolism.

(A) Lipids from the plasma membrane are degraded after endocytosis and internalization into intraendosomal luminal vesicles (IEV) and intralysosomal luminal vesicles (ILV). Lysosomal glycosphingolipids are degraded in a stepwise manner. (B) Structure of the anionic phospholipid BMP, (C) endolysosomal phospholipid degradation of sphingomyelin by acid sphingomyelinase, a phospholipase C, and of phosphatidylcholine by the lysosomal phospholipase A2. ASM, Acid sphingomyelinase; BMP, bis(monoacylglycerophosphate); Cer, ceramide; Chol, cholesterol; GlcCer, glycosylceramide; IEV, intraendosomal luminal vesicles; ILV, intralysosomal luminal vesicles; lyso-PC, lyso-phosphatidylcholine; NPC, Niemann-Pick disease type C protein; PC, phosphatidylcholine; PLA2, phospholipase A2; PM, plasma membrane; S1P, sphingosine-1-phosphate; SAP, sphingolipid activator protein; SM, sphingomyelin; So, sphingosine.

have to assume that the surface of ILVs is the main location for the enzymatic breakdown of complex amphiphilic lipids in the lysosomal compartment (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019).

Lipids of the surrounding lysosomal perimeter membrane are protected from degradation (Henning and Stoffel, 1973) by a thick glycocalix surrounding the membrane facing the lysosol (Figure 2A) (Eskelinen et al., 2003; Breiden and Sandhoff, 2019). It is generated by integral lysosomal membrane glycoproteins, which are heavily N-glycosylated with digestion resistant polygalactosamine oligosaccharide chains. It forms an efficient hydrophilic barrier at the luminal surface of the lysosomal perimeter membrane to prevent its degradation by proteases and hydrolases. It also is an efficient barrier for lipids like

phospholipids, sphingolipids, cholesterol and most of their hydrophobic breakdown products preventing them from reaching the lysosomal perimeter membrane and escaping from the lumen of the lysosome (Breiden and Sandhoff, 2019).

Whereas most proteins are protonated and positively charged at the low pH values prevailing in the lysosomal compartment, the ILVs are negatively charged, mainly due to the generation of the anionic lysolipid bis(monoacylglycerol)phosphate (BMP) (Figure 2B) in the ILV membranes as an intermediate of the phosphatidylglycerol catabolism. *In vitro* experiments demonstrate that 20 mol% of BMP (or of another anionic phospholipid like phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, etc.) generate a negative net charge on the

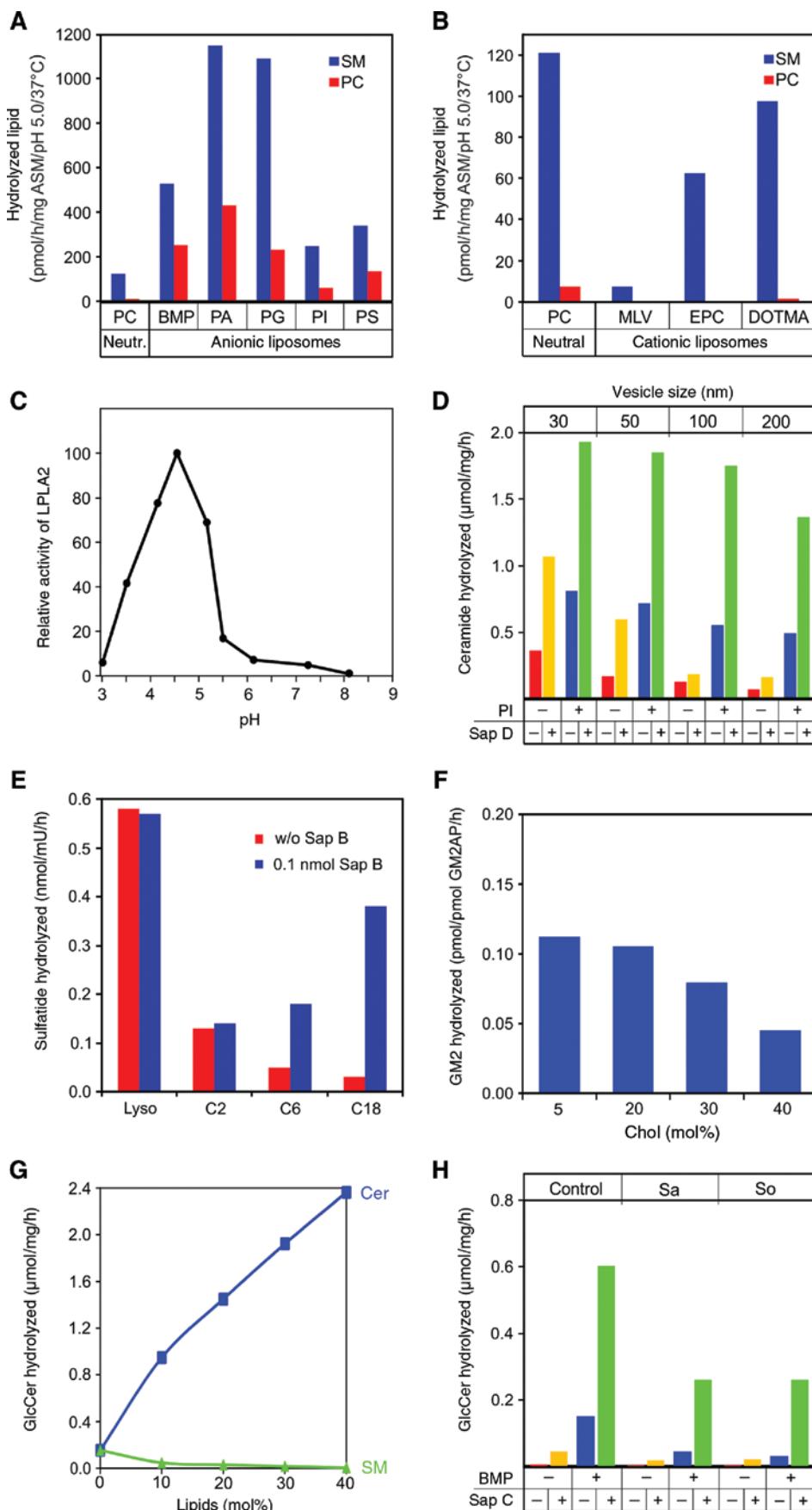


Figure 3: Phospholipid degradation is regulated by lipid composition of the vesicles (A, B) and the pH (C). The presence of anionic lipids (BMP, PA, PG, PI, PS) enhances the degradation of sphingomyelin and phosphatidylcholine by acid sphingomyelinase, a phospholipase C. On the other hand, cationic substances [cationic lipids (B) or cationic amphiphilic drugs] inhibit the digestions of these phospholipids. (Glyco)Sphingolipid degradation decreases with vesicle size (D) and acylchain length of lipids in the absence of Sap B (E). The hydrolysis of (glyco)sphingolipids is stimulated by sphingolipid activator proteins [(D) Sap D enhances hydrolysis of short ceramide by acid ceramidase, (E) Sap B the hydrolysis of sulfatide by the ASA, (H) Sap C the hydrolysis of GlcCer by GBA1] and by anionic lipids and ceramide (G). The presence of cholesterol (F), sphingomyelin (G), and of cationic substances, and of sphingoid bases sphingosine and sphinganine (H) inhibits the digestions of (glyco)sphingolipids.

Data are from the following publications: (A, B) (Oninla et al., 2014), (C) (Abe and Shayman, 2009), (D) (Linke et al., 2001), (E) (Vogel et al., 1991), (F) (Anheuser et al., 2015), (G, H) (Abdul-Hammed et al., 2017). BMP, Bis(monoacylglycerophosphate); Cer, ceramide; Chol, cholesterol; DOTMA, 1,2-di-O-octadecenyl-3-trimethylammonium propane; EPC, 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine; GBA1, lysosomal β -glucocerebrosidase; GlcCer, glucosylceramide; GM2AP, GM2 activator protein; LPLA2, lysosomal phospholipase A2; MVL 5, multivalent cationic lipid; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; Sa, sphinganine; Sap, saposin; So, sphingosine.

vesicle surface even at lysosomal pH values (Oninla et al., 2014). Due to its unusual chemical configuration (*sn*1 and S-configuration instead of the R-configuration of the normal membrane-forming phospholipids), BMP is catabolized slowly and therefore increases up to 40–60 mol% of the phospholipids in the ILVs (Kobayashi et al., 1999; Gallala et al., 2011). Like phosphatidylglycerol and phosphatidylinositol, BMP carries a negative net charge, even at pH values as low as 5, and acts as an efficient stimulator of sphingolipid catabolism at ILV-surfaces (Wilkening et al., 1998; Oninla et al., 2014).

In vitro studies suggest that anionic phospholipids like BMP, concentrating in ILV membranes (Möbius et al., 2003), generate a negative surface potential on ILVs, which should electrostatically attract and bind positively charged cationic lysosomal hydrolases (Kölzer et al., 2004; Oninla et al., 2014) and sphingolipid activator proteins (SAPs) (Locatelli-Hoops et al., 2006; Remmel et al., 2007; Anheuser et al., 2015; Graf et al., 2017) on the surface of ILVs and thereby speed up the catabolic rates of ILV-bound lipids.

As observed for surface bound acid sphingomyelinase (Hurwitz et al., 1994), acid ceramidase (Elojeimy et al., 2006), β -hexosaminidase A (Hex A) and Hex B (and presumably also relevant for many other surface-bound glycoproteins like phospholipases and most other lysosomal hydrolases), these highly glycosylated hydrolases are partially protected against premature proteolytic digestion in late endosomes and lysosomes when bound to the ILV surface. However, compensation of the negative surface charge of the ILVs by incorporation of a CAD (Kölzer et al., 2004), for example, after feeding of the anti-depressant desipramine into cultured fibroblasts, should favor a release and proteolytic digestion of many cationic hydrolases and other glycoproteins, as demonstrated for acid sphingomyelinase (Hurwitz et al., 1994). This reduces the catabolism of ILV-bound lipid substrates, triggering an

induced phospholipid- and sphingolipid storage disease and possibly also a neutral lipid storage disease, in other words, an induced phospholipidosis (Lüllmann et al., 1978; Hurwitz et al., 1994; Kölzer et al., 2004).

Maturation of ILVs by sorting of membrane lipids during endocytosis

The surrounding membranes of organelles in eukaryotic cells maintain an organelle-specific lipid and protein composition (van Meer et al., 2008). The cellular plasma membranes are usually enriched in stabilizing lipids, having a high content of cholesterol (approximately up to 40 mol% of all membrane lipids), as well as sphingomyelin and complex glycosphingolipids in their outer lipid layer. Both lipids, sphingomyelin and cholesterol, turned out to be major inhibitors of key steps in lysosomal sphingolipid catabolism (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019).

The simultaneous degradative clearance of inhibitory sphingomyelin and concomitant increase of activating ceramide level by the action of acid sphingomyelinase (Figure 2C) stimulate several steps in sphingolipid catabolism (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019). Also the removal of cholesterol from the nascent ILVs membranes by the two sterol binding and transfer proteins NPC1 and NPC2 along the endocytotic pathway is essential to enable several key steps of endolysosomal sphingolipid degradation (Figure 2) (Abdul-Hammed et al., 2010; Wang et al., 2010; Oninla et al., 2014; Anheuser et al., 2015) as well as in the phospholipid degradation by lysosomal phospholipase A1 (Piret et al., 2005).

Indeed, induced sphingomyelin accumulation in acid sphingomyelinase deficient Niemann-Pick disease types

A and B cells and tissues inhibits cholesterol export by NPC2 from the ILVs and triggers a secondary accumulation of cholesterol as well as a mild accumulation of small gangliosides (GM2 and GM3) and glycolipids like glucosylceramide in the lysosomal compartment of patients' tissues and cells with Niemann-Pick types A and B. Patients with Niemann-Pick disease type C primarily accumulate cholesterol and secondarily also sphingomyelin in the lysosomes due to an inherited defect of a cholesterol transfer protein, either NPC1 or NPC2 (Vanier, 1983; Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019). These accumulating lipids also inhibit the activity of the sphingolipid activator proteins studied so far, like GM2 activator protein, Sap A and Sap B (Locatelli-Hoops et al., 2006; Remmel et al., 2007; Anheuser et al., 2015, 2019b), attenuating the catabolism of further sphingolipids.

Therefore, a decrease of cholesterol and sphingomyelin levels as well as the generation of anionic BMP and ceramide in the membranes of the ILVs during their maturation seems to be necessary to allow physiological catabolic rates of several gangliosides, glycosphingolipids, sphingolipids and presumably also of other complex lipids.

The lysosomal catabolism is also strongly affected by other factors in the micro-environment of the enzymatic reaction (Figure 3). The major influence of pH value, vesicle size, temperature and ionic strength is well known (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019). The activity of lysosomal lipases, phospholipases, glycosidases, and other proteases is presumably also strongly affected by the nature of their substrates, especially as demonstrated for the turnover of various glycolipid substrates by hexosaminidases. These glycohydrolases are rather promiscuous enzymes that degrade a wide range of oligosaccharides, glycoproteins, glycosaminoglycans besides glycolipids by cleaving off β -glycosidically linked terminal *N*-acetylglucosaminide, *N*-acetylgalactosamide and *N*-acetylglucosaminide-6-sulfate residues (Sandhoff et al., 1977; Hepbildikler et al., 2002; Sandhoff and Harzer, 2013). For example, soluble artificial substrates are cleaved by Hex A within a broad pH profile (3–7), the hydrolysis of micellar glycolipid GA2 occurs in a narrow pH range (3.5–5) in the presence of detergents (Sandhoff et al., 1971), whereas the degradation of the membrane-bound ganglioside GM2 in the presence of the natural activator protein GM2 activator protein is achieved only within a very narrow pH range (3.8–4.6) (Bierfreund et al., 1999), and is optimally stimulated by a negative surface charge of the membranes in the presence of ceramide (Anheuser et al., 2019b).

In contrast to soluble artificial substrates often used to measure lysosomal hydrolases by *in vitro* assays, their

physiological lipid substrates are insoluble and are often components of biological membranes or other lipophilic phases *in vivo*. In this case, even the acyl chain length of lipids affects the enzymatic lipid turnover, as shown for the cleavage of sulfatides by arylsulfatase A (Vogel et al., 1991).

Furthermore, the catabolic rates of complex lipid-substrates that are components of ILVs are heavily affected by the presence or absence of SAPs and by the presence and absence of stimulatory and/or inhibitory lipids in the lipid substrate carrying membranes (Figure 3) (Breiden and Sandhoff, 2019). It is expected that the catabolic rates of phospholipids as catalyzed by lysosomal cationic phospholipases (phospholipase A1 and A2, acid sphingomyelinase) (Shayman and Abe, 2013; Oninla et al., 2014; Shayman and Tesmer, 2019) and of triglycerides, diglycerides and cholesterol esters as mediated by cationic lysosomal lipases, are also strongly modified by electrostatic binding of these protonated and positively charged enzymes to the negatively charged lipid substrate carrying surfaces. Both major phospholipases, lysosomal phospholipase A2 (Glukhova et al., 2015) and acid sphingomyelinase (Breiden and Sandhoff, 2019), are attracted by anionic lipids and bind to negatively charged membrane surfaces.

The turnover of all these lipids might be stimulated by the incorporation of anionic BMP and other anionic lipids into the substrate carrying membrane and/or micelle surfaces (Piret et al., 2005; Abe and Shayman, 2009; Shayman and Abe, 2013; Oninla et al., 2014) and inhibited by the insertion of cationic amphiphiles like sphingoid bases or synthetic positively charged amphiphiles as demonstrated for the catabolism of some sphingolipids and glycosphingolipids (Anheuser et al., 2019a,b; Breiden and Sandhoff, 2019). The activity loss of acid sphingomyelinase, a lysosomal phospholipase C in patients and knock-out mice, and of lysosomal phospholipase A2 in mouse models (Hiraoka et al., 2006; Abe and Shayman, 2009) caused a phospholipid accumulation and resulted in the appearance of intracellular lamellar bodies as observed in CAD induced phospholipidosis. Both enzyme activities are inhibited by CADs and stimulated by anionic lipids (Abe and Shayman, 2009; Oninla et al., 2014).

The lysosomal digestive system is in a steady state homeostatic equilibrium between the influx of macromolecules including complex lipids, the catabolic turnover rates and the secretion of degradation products like free fatty acids, hexoses and other small molecular carbohydrates.

We suppose that the activity of phospholipid and sphingolipid binding and solubilizing activity of SAPs

together with the generation of micelle forming and bilayer destroying lipids (free fatty acids, lysophospholipids, di- and monoglycerides) by the action of phospholipases and acid sphingomyelinase will readily destroy the lipid bilayer structure of ILV membranes, dissolve the ILVs and generate a mix of micelles and other lipid aggregates. The generation of new lysosomes and their dysfunctional disappearance as residual bodies is probably also in a homeostatic equilibrium including the formation of intraendosomal vesicles, their maturation at the level of late endosomes, for example, by the removal of inhibitory membrane lipids like cholesterol and sphingomyelin and their final degradation in healthy cells.

Mechanisms of drug-induced phospholipidosis

DIPL is a lysosomal storage disorder characterized by excessive accumulation of phospholipids and CADs in the lysosomes and the formation of lamellar bodies (Figure 1). It resembles the phospholipid accumulation of the lysosomal storage disease Niemann-Pick disease type A and B, which is caused by deficient activity of the acid sphingomyelinase, triggering an endolysosomal storage of sphingomyelin (Figure 2C), and a secondary accumulation of unesterified cholesterol, BMP, and small gangliosides and glycosphingolipids (Vanier, 1983; Schuchman and Desnick, 2001; Oninla et al., 2014; Breiden and Sandhoff, 2019). Furthermore, knock-out mice deficient in lysosomal phospholipase A2 show an accumulation of phospholipids, in particular phosphatidylethanolamine and phosphatidylcholine in alveolar and peritoneal macrophages, and the spleen (Hiraoka et al., 2006). A phospholipidosis-like phenotype has also been observed in some inherited lysosomal storage diseases, especially in cholesterol storage disease and in Sandhoff disease (Lecommandeur et al., 2017).

DIPL is induced by CADs, which can trigger an excessive, but reversible accumulation of phospholipids and drugs in lysosomes. CADs were thought to increase lysosomal pH and reduce lysosomal enzyme activity. CADs can raise the pH value of the endolysosomal compartments toward neutrality (Goldman et al., 2009) as shown by the *in vivo* administration of chloroquine (Tietz et al., 1990). It causes a limited and transient increase of the lysosomal pH (Tietz et al., 1990; Zheng et al., 2011; Hamaguchi et al., 2014). The endolysosomal pH is mainly regulated by vacuolar ATPase (v-ATPase). Hamaguchi et al. could show that a long CAD treatment did not inhibit the v-ATPase and

maintained the endolysosomal pH homeostasis (Hamaguchi et al., 2014).

The reduction of lysosomal hydrolase activities, however, seems to be mainly caused by blockade of mannose-6-phosphate receptor mediated targeting of lysosomal enzymes and SAPs by CADs. CADs also cause an alternation of the lysosomal membrane composition, a modulation of the lysosomal signaling and trigger lipid accumulation and the formation of lamellar bodies (Figure 4) (Sandra and Diego Luis, 2017). Close similarities have also been observed between the lipid profile of liver tissues of Sandhoff mice and the drug induced phospholipidosis in rats, including an increase in BMPs, cholesterol esters, lysophosphatidylcholine levels and a higher percentage of long chain ceramides (Lecommandeur et al., 2017). An increase of BMPs, especially of di-22:6-BMP, was also observed in DIPL (Liu et al., 2014) and is now used as a biomarker for DIPL, which can be detected by liquid chromatography tandem mass spectrometry (LC-MS/MS) in plasma/serum, urine and tissue samples.

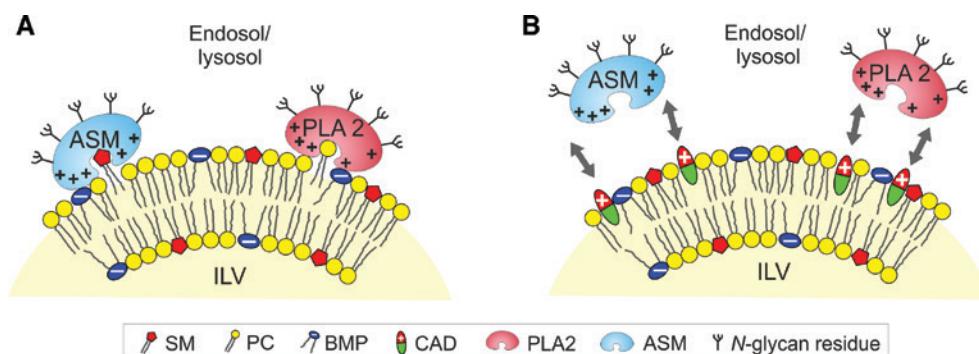
So far, it remains unclear if CADs in general can bind endosomal and lysosomal proteins directly and thereby inhibit their functions and the digestive capacity of the lysosome. It has been shown, however, that the antibiotic gentamicin binds to megalin, a glycoprotein receptor of the early endosomal pathway of proximal tubular cells in the kidney, that delivers the CADs to the lysosomes (Hammond et al., 1997). It induces a vacuolation associated with an inhibition of early fusion of endosomes, damaging the endosomal pathway. A moderate inhibition of β -glucosidase and β -galactosidase was observed by chloroquine (Harder et al., 1985).

Emerging mechanisms of inducing a ‘phospholipidosis’ by CADs

Cellular uptake of CADs and their accumulation in the acidic compartments of the cell

CADs are amphiphilic molecules that contain a hydrophobic, often aromatic ring structure and a slightly hydrophilic head-group that can be protonated at its weak base, usually a primary, secondary or tertiary nitrogen-atom N bound to a carbon C of an alkyl chain (Table 1).

The unprotonated neutral CAD molecules are rather hydrophobic and can penetrate biological membranes. They can be taken up by cells, either by this mechanism, or possibly also by endocytotic and pinocytotic mechanisms.



Lipid degradation in the endolysosomal compartment

Key steps facilitating physiological lipid catabolism:

- Degradation of inhibiting SM thereby generation of stimulatory ceramide
- Cholesterol transfer out of the late endosomes
- Generation of BMP

CADs alter lysosomal functions:

- By lowering electrostatic binding of enzymes to the lipid substrate-carrying membranes
- Proteolytic digestion of lysosomal hydrolases
- Block of M6PR-mediated targeting of lysosomal hydrolases
- Reduction of lipid cleaving activity of enzymes
- Accumulation of storage material
- Formation of lamellar bodies
- Block of Ca^{2+} -signaling
- Permeabilization of lysosomal membranes followed by cell death

Figure 4: Model of the electrostatic interaction of ASM and PLA2 with intraendolysosomal vesicles (ILV) of untreated (A) and CAD-treated cells (B).

CADs contain a hydrophilic amine head group that can be protonated at physiological pH (red) and a hydrophobic tail consisting of an aromatic or aliphatic ring structure (green). This is the reason that CADs accumulate progressively in the endolysosomal compartment. +, Positively charged; -, negatively charged; ASM, acid sphingomyelinase; BMP, bis(monoacylglycerophosphate); CAD, cationic amphiphilic drug; ILV, intraendolysosomal luminal vesicles; M6PR, mannose-6-phosphate receptor; PLA2, phospholipase A2; PC, phosphatidylcholine; SM, sphingomyelin.

The uptake mechanism of CADs to enter the membranes of the endolysosomal system is called lysosomotropism or lysosome trapping (de Duve et al., 1974; Trapp et al., 2008). The efficiency of this mechanism depends on the chemical properties of the drug (Trapp et al., 2008; Rhein et al., 2018). Two parameters are important for an effective cationic amphiphilic character of a molecule:

- a) A high basic pK_a value (logarithmic acid dissociation constant) >7.4 , which indicates that these substances are mainly protonated at low pH values
- b) The $c\log P$ value (a logarithmic partition coefficient) in the range of 2 and 9 (Rhein et al., 2018). It describes the amphiphilicity of charged molecules, and is a coefficient of their lipid solubility, a measure for their distribution between a hydrophilic and hydrophobic phase (mostly used is a water/octanol system).

These parameters are used to estimate if a substance is a lysosomotropic compound and is able to induce a DIPL (Table 1).

As soon as the CADs reach an acidic compartment (Halliwell, 1997), endosomes or lysosomes, they become protonated, and as positively charged amphiphiles they are trapped within the acidic compartment, where they accumulate with time (MacIntyre and Cutler, 1988; Koda-vanti et al., 1990; Daniel et al., 1995).

Due to electrostatic attraction as shown by *in vitro* experiments (Kölzer et al., 2004; Anheuser et al., 2015), they can bind to the anionic surfaces of intraluminal vesicles of the late endosome and lysosome and presumably can even insert with their hydrophobic moiety into the lipid bilayer structure, thereby reducing and eventually compensating the negative surface charge of the ILVs.

CADs disturb the central role of ILVs for catabolism of complex lipids

The increasing accumulation of CAD-molecules at the surface of ILVs will reduce their negative surface potential created by anionic phospholipids like BMP and others

(phosphatidic acid, phosphatidylglycerol, phosphatidylinositol, etc.), even at pH values as low as 5 (Oninla et al., 2014), and thereby release lysosomal hydrolases and SAPs from the ILV surfaces (Kölzer et al., 2004; Anheuser et al., 2015) (Figure 4). A release of the catabolic hydrolases and SAPs from the phospholipid and sphingolipid carrying ILV membranes should cause a drastic reduction of the lipid catabolism (Oninla et al., 2014; Anheuser et al., 2015; Abdul-Hammed et al., 2017). The released phospholipases, lipases, glycosidases and sphingolipid cleaving enzymes should be easy prey for lysosomal proteases, as shown for acid sphingomyelinase which is a lysosomal phospholipase C (Hurwitz et al., 1994), thereby reducing the catabolic potential of the lysosomal compartment further, causing an increasingly complex endosomal and lysosomal storage disease. Depending on the structural properties of the individual CADs used and their doses applied, a heterogenic accumulation of hydrophobic and amphiphilic molecules is expected, including mainly phospholipids and glycerolipids, sphingolipids and other hydrophobic materials like insoluble protein fragments and cholesterol. The secretion of cholesterol might well be reduced by inhibitory phospholipids such as sphingomyelin that inhibits cholesterol export by NPC2 from ILVs and catabolism of GM2, glucosylceramide and others (Oninla et al., 2014; Anheuser et al., 2015; Abdul-Hammed et al., 2017). Sphingomyelin effectively blocks cholesterol export by NPC2 from the lysosomal compartment (Babalola et al., 2007; Oninla et al., 2014) and triggers as a primary storage compound in Niemann-Pick disease types A and B patients an excessive secondary storage of cholesterol. Both storage compounds, sphingomyelin and cholesterol, inhibit catabolic processes of small gangliosides (GM2 and GM3) and glycosphingolipids like glucosylceramide (Anheuser et al., 2015; Abdul-Hammed et al., 2017; Sandhoff and Sandhoff, 2018). Also, levels of other molecules like insoluble hydrophobic protein fragments may increase in the forming lamellar bodies, such as the membrane-spanning hydrophobic fragments of ATP synthase (Palmer, 2015), which are soluble in lipid phases and hardly digestible.

Accumulating CADs inhibit lysosomal lipid catabolism effectively

Due to the electrostatic binding of cationic lysosomal proteins (lipases, phospholipases, sphingolipid degrading hydrolases, SAPs and others) to the anionic surfaces of ILVs (Figure 4), uptake of CADs will trigger a compensation of the negative surface charge and release most of the

catabolic proteins from the lipid substrate carrying surfaces of the ILVs (e.g. sphingolipid and glycosphingolipid degrading hydrolases and activator proteins (Sandhoff and Sandhoff, 2018), phospholipases A1, A2 (Abe and Shayman, 2009) and phospholipase C, the acid sphingomyelinase (Oninla et al., 2014; Sandhoff and Sandhoff, 2018) and lipases). The following proteolytic digestion of the released lysosomal hydrolases (Hurwitz et al., 1994) and SAPs should strongly reduce the catabolism of most lipids (phospholipids, sphingolipids, cholesterol esters and neutral glycerolipids) and decrease the overall catabolic potential of the lysosomes further, triggering the conversion of affected lysosomes to dysfunctional storage granules, the lamellar bodies.

Eventually, the continuous cellular uptake of CADs and their incorporation into ILVs will trigger a fatal sequence, a reduction of the negative net surface charge of the ILVs, a release of the phospholipid, sphingolipid and lipid catabolizing hydrolases from the surfaces of ILVs and thereby a drastic reduction of the catabolic degradation of ILV-bound lipids as well as a strongly attenuated cholesterol export from the lysosomes (Oninla et al., 2014; Abdul-Hammed et al., 2017; Breiden and Sandhoff, 2019). This will trigger the secondary accumulation of additional inhibitory lipids like sphingomyelin and cholesterol. The catabolic potential of the lysosomal system will be further strongly impaired by the proteolytic digestion of lysosomal hydrolases released from the ILV surfaces (Hurwitz et al., 1994).

In summary, the manifold accumulation of lipids and hydrophobic material in the lysosomes will trigger a vicious cycle, a snowball system that will bring the degradative function of the lysosomes at a halt like a downstream vortex, so that they will end as dysfunctional lamellar bodies.

CADs inhibit the lysosomal degradation of major lipids, sphingolipids, phospholipids and presumably also of neutral lipids by directly affecting the functions of ILVs, the common platform of cellular lipid catabolism. As observed, the resulting lysosomal storage is dominated by the accumulation of phospholipids, as their normal turnover exceeds that of sphingolipids.

Sphingolipids are degraded in a strict sequential and stepwise manner; an interruption of a single step blocks the entire chain and causes an often fatal accumulation of its substrates (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019). The excessive sphingomyelin accumulation in Niemann-Pick disease induced by acid sphingomyelinase deficiency, however, additionally triggers the secondary lysosomal accumulation of several lipids, cholesterol by inhibiting its secretion by NPC2 and several

catabolic steps of small glycosphingolipids like glucosylceramide, and small gangliosides like GM2 by inhibiting their catabolism, as discussed already.

Many known phospholipases, however, can attack their phospholipid and lysophospholipid substrates independently of each other (Wilton and Waite, 2002). Thus, phospholipid degradation does not occur in an ordered sequence of steps as observed for sphingolipid catabolism, and thereby an excessive lipid accumulation can be avoided in the case of a single phospholipase deficiency. A phospholipase deficiency in a knock-out mouse, for example, can be bypassed at least in part by other phospholipases avoiding a complete block of phospholipid degradation and a fatal storage disease (Hiraoka et al., 2006).

CADs can induce cell death in tumor cells

Lysosomes are involved in several cellular processes like defense of pathogens, membrane repair, signaling and cell death (Pu et al., 2016). Though lysosomotropic CADs induce a phospholipidosis, they are still used for the treatment of depression, allergies and hypertension. As reported recently, they may also have an antitumor effect, preferentially killing the more sensitive cancer cells by releasing lysosomal cathepsins into the cytoplasm (Petersen et al., 2013).

Tumor cells contain more and larger lysosomes and have an enhanced cathepsin activity compared to normal cells (Saftig and Sandhoff, 2013). CAD treatment leads to a decreased acid sphingomyelinase activity (Albouz et al., 1986) and an enhanced cell death in tumor cells (Petersen et al., 2013). CADs can displace acid sphingomyelinase from the ILVs (Figure 4B) and trigger its proteolytic degradation (Hurwitz et al., 1994), which leads to enhanced sphingomyelin levels in lysosomal membranes. This is thought to induce lysosomal membrane permeabilization and release of cathepsins into the cytosol (Figure 4), triggering cell death (Kirkegaard et al., 2010; Petersen et al., 2013).

Conclusion and outlook

Our favored concept for the development of an induced phospholipidosis originates from studies on sphingolipid and phospholipid catabolism, on the cell biology of sphingolipidoses and the characterization of lysosomal phospholipases, acid sphingomylinase, which is basically a phospholipase C, and phospholipase A2.

So far, no evidence has been presented for alternative mechanisms explaining the common capability of

more than 50, structurally very different CADs, to induce phospholipidosis. There is no compelling evidence for an enhanced biosynthesis of cholesterol or phospholipids available, nor for a direct and effective inhibition of lysosomal phospholipases by all these drugs or an induction of non-degradable drug-phospholipid complexes.

Central for our concept is the accumulation of CADs in the endosomal and lysosomal compartments, the direct interference of CADs with the ILVs, the cellular platforms for sphingolipid and phospholipid degradation, and at the end a possible increase of pH values (e.g. by attenuating the vacuolar proton pump during formation of multi-lamellar bodies, MLBs).

CADs can concentrate and progressively accumulate in the lysosomes, thereby competing with the lipid cleaving hydrolases for binding to the negatively charged surfaces of ILVs. They can also insert into ILV-membranes and thereby compensate their negative surface charge, which, however, is essential for physiological rates of lysosomal lipid catabolism, as it attracts protonated and positively charged catabolic hydrolases and SAPs to the site of lipid catabolism at the ILV-surfaces. CADs can thereby release the lipid splitting glycoprotein-hydrolases from the lipid substrate carrying ILV surfaces into the lysosol, where they become prey for proteolytic digestion (Hurwitz et al., 1994; Kölzer et al., 2004).

CADs reduce the digestive potential of the lysosomes, especially for the insoluble, ILV-bound and most abundant phospholipids, but also for the less abundant sphingolipids and neutral lipids. The progressively accumulating lipids and CADs can aggregate in more and more dysfunctional lamellar bodies generating effects in DIPL that are similar to pathologic features observed in some lysosomal storage diseases. In healthy mammalian tissues, however, ILVs are themselves digested almost completely together with their cargo of complex lipids and other macromolecules like proteins.

Jamming the endosomal and lysosomal compartment with an ever increasing storage of CADs and an increasing variety of lipids will eventually impair and block most of the lysosomal functions, especially their digestive capacity as stomachs of the cell to release nutrients into the cytosol and function as a signaling organelle for cellular energy metabolism (Settembre et al., 2013; Pastore et al., 2017). By aging and dysfunctional storage, lysosomes are eventually converted to almost undegradable MLBs (Figure 1).

As a last resort, cells may generate new lysosomes to keep their metabolic turnover running. They may even regenerate cellular metabolism as soon as the uptake of CADs has been stopped. Therefore, DIPL can be reversible

to some extent in contrast to the irreversible loss or functional impairment of a single lipid degrading hydrolase in genetic storage diseases.

The hypothesis presented here focuses on general and common properties of a vast variety of chemically different CAD structures, which are sufficient to trigger a DIPL. A main feature is their amphiphilic nature and their positive charge at low pH values, which allows them (A) to compete with the positively charged lysosomal proteins at the negatively charged ILV surfaces for binding and (B) to integrate into the membranes of the ILVs, to compensate for their negative surface charge.

Thereby, they release lysosomal enzymes and SAPs into the lysosol where they are presumably destroyed by proteolysis as demonstrated for the main phospholipase C, the acid sphingomyelinase and a few other hydrolases (Hurwitz et al., 1994).

CADs can trigger the secretion of lysosomal enzymes from the cells (Ikeda et al., 2008), reducing the degradative potential, thereby switching lysosomes into dysfunctional MLBs storing a multitude of undegraded complex lipids, CADs and also lipophilic protein fragments (MacIntyre and Cutler, 1988; Kodavanti and Mehendale, 1990; Lecommandeur et al., 2017).

The wide range of complex lipids stored differs significantly from that of sphingolipidoses caused by the defect of a single catabolic step. It has however, some similarities to sphingolipidoses caused by defects in more than one deficient hydrolase (e.g. Sandhoff disease), or by the loss of acid sphingomyelinase activity, since its primary storage compound, sphingomyelin, inhibits many catabolic pathways simultaneously (Sandhoff and Sandhoff, 2018; Breiden and Sandhoff, 2019).

Our hypothesis focuses on the disturbance of ILVs, which play a key role in lipid catabolism. Their central function is cut off by the accumulation of CADs, which will eventually convert them into dysfunctional and degradation resistant MLBs. It does not elucidate and specify the contribution of most of the many single hydrolases during the CAD induced progressive conversion of functional lysosomes into dysfunctional granules.

Studies on the biosynthesis and degradation of lysosomal hydrolases in the presence of a CAD, desipramine, indicate that the main phospholipases (e.g. acid sphingomyelinase) in cultured fibroblasts are readily inactivated and degraded (Kirkegaard et al., 2010; Petersen et al., 2013; Saftig and Sandhoff, 2013). Their loss should attenuate the generation of bilayer destroying and micelle forming free fatty acids and lysophospholipids thereby opening the path for the conversion of functional lysosomes into dysfunctional MLBs, basically inactive storage granules.

Their accumulation and the loss of functional lysosomes, must have a lasting limiting and toxic effect on cellular energy metabolism and anabolism (Settembre et al., 2013; Pastore et al., 2017), the details of which are still open for investigation.

It remains unclear, which impact each of the simultaneously dying off catabolic pathways may have on the CAD-induced immuring of hydrolases, CADs and lipid substrates during the conversion of lysosomes into dysfunctional MLBs. There are no comparable quantitative data available to clarify which of the concurrently running out pathways and which hydrolases are more important than others, or which one could be sufficient by themselves. Detailed and quantitative information is also missing on the lipid pattern of individual DIPLs and their correlation as caused by structurally different CADs. It remains an open question to which extent the impact of the functional loss of individual hydrolases differs during the switch from functional lysosomes to MLBs. Among the hydrolases studied in our lab, acid sphingomyelinase was degraded proteolytically faster than others (Hex A, acid ceramidase). Early loss of its activity should accumulate its main substrates, especially sphingomyelin and various other phospholipids within the lysosomes. Accumulating sphingomyelin is known to inhibit cholesterol secretion by the NPC2 protein and, therefore, to trigger a massive secondary cholesterol accumulation in the lysosomes, as observed in Niemann-Pick disease type A and B (Vanier, 1983). Both storage compounds attenuate the catabolism of other lipids like glucosylceramide (Abdul-Hammed et al., 2017) and the action of essential SAPs (GM2 activator protein, Sap A, Sap B) (Locatelli-Hoops et al., 2006; Remmel et al., 2007; Anheuser et al., 2015, 2019a,b). This might be a reason for similarities in the lipid storage pattern of Niemann-Pick diseases and DIPL.

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