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## Niemann-Pick type C disease: molecular mechanisms and potential therapeutic approaches

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### Abstract

Cholesterol is an important lipid of mammalian cells. Its unique physicochemical properties modulate membrane behavior and it serves as the precursor for steroid hormones, oxysterols and vitamin D. Cholesterol is effluxed from the late endosomes/lysosomes via the concerted action of at least two distinct proteins: Niemann-Pick C1 and Niemann-Pick C2. Mutations in these two proteins manifest as Niemann-Pick type C disease – a very rare, usually fatal, autosomal, recessive, neurovisceral, lysosomal storage disorder. In this review we discuss the possible mechanisms of action for NPC1 and NPC2 in mediating cholesterol efflux, as well as the different therapeutic approaches being pursued for the treatment of this lipid storage disorder.

### Keywords

cyclodextrin; miglustat; acid sphingomyelinase; lysosomal storage organelle; lysosomal acid lipase; bis(monoacylglycerol)phosphate

### Overview

Cholesterol is a critical lipid of mammalian cells. Its unique physicochemical properties modulate membrane behavior and it serves as the precursor for steroid hormones, oxysterols and vitamin D and its deregulation affects numerous pathological processes. In the peripheral tissues of mammals there are two sources of cholesterol. Cholesterol can be supplied exogenously, being delivered to the cells as part of receptor-mediated lipoprotein uptake. Alternatively, cholesterol can be generated via *de novo* synthesis in the endoplasmic reticulum (ER). In the central nervous system (CNS), the latter pathway is utilized exclusively due to the inability of LDL particles to cross the blood-brain barrier. Free cholesterol synthesized in the glia and complexed with apolipoprotein E (ApoE) is delivered to neurons to supplement their endogenous sterol production (Dietschy & Turley 2004). Cholesterol homeostasis is integrated at the cellular level by the tight regulation via the SCAP/INSIG/SREBP sterol sensing system. In the peripheral tissues, after lipoprotein delivery to late endosomes/lysosomes (LE/LY), cholesterol is liberated from cholesteryl esters by lysosomal acid lipase (LAL, EC 3.1.1.3), an acidic hydrolase that also cleaves triacylglycerides and is part of the lipase family that includes gastric and lingual lipases (Anderson & Sando 1991). LAL is produced as a proenzyme that is targeted to the LE/LY via mannose-6-phosphate modification (Sando & Henke 1982), and its deficiency results in either Wolman disease or its milder variant, Cholesteryl Ester Storage Disease (CESD)

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(Sloan & Fredrickson 1972, Assmann 1995). The difference in the severity of the phenotype between CESD and Wolman disease originates primarily from the levels of residual enzymatic activity of LAL, depending on the molecular nature of the mutation (Anderson *et al.* 1999). Enzyme replacement therapy has been pursued as a treatment option for Wolman/CESD (Du *et al.* 2001). In addition, LAL has been suggested to play a significant role in atherosclerosis (Du & Grabowski 2004, Du *et al.* 2004, Grosheva *et al.* 2009, Haka *et al.* 2009), thus generating further interest in elucidating its function in cells and obtaining tools (e.g., specific small molecule inhibitors) for such studies (Rosenbaum *et al.* 2010a).

Cholesterol is effluxed from the LE/LY via the concerted action of at least two distinct proteins: Niemann-Pick C1 (NPC1) (Carstea *et al.* 1997) and Niemann-Pick C2 (NPC2) (Naureckiene *et al.* 2000). In normal cells, cholesterol is exported from the LE/LY and delivered to other organelles, including the plasma membrane, the endocytic recycling compartment and the endoplasmic reticulum (Mesmin & Maxfield 2009). The exact nature of intracellular cholesterol transport is an area of active investigation (Mesmin & Maxfield 2009), with both vesicular and non-vesicular transport mechanisms playing a role (Maxfield & Wustner 2002, Hao *et al.* 2002). Elevated levels of cholesterol in the ER lead to increased esterification by acyl Co-A:cholesterol acyl transferase (ACAT) (Goldstein *et al.* 1974). Cholesterol esters formed by ACAT are stored as lipid droplets that are hydrolyzed by cytoplasmic neutral cholesterol ester hydrolase (Okazaki *et al.* 2008, Small *et al.* 1989). Cellular cholesterol can also be exported to extracellular acceptors, such as high density lipoproteins, in a process mediated by ATP-binding cassette transporters such as ABCA1 (Tall 2003).

Mutations in the two NPC proteins manifest as NPC disease – a rare, usually fatal, autosomal, recessive, lysosomal storage disorder. In both types of NPC disease cholesterol, bis(monoacylglycerol)phosphate (BMP), and various sphingolipids accumulate in specialized compartments, lysosomal storage organelles (LSOs), leading to altered protein and lipid trafficking (Pipalia *et al.* 2007, Choudhury *et al.* 2004). While most of the evidence points to both LDL-derived (Pentchev *et al.* 1985) and non-LDL-derived (Pipalia *et al.* 2007) cholesterol as the primary storage metabolite in NPC disease, especially in the peripheral tissues, other possibilities include glycosphingolipids, sphingomyelin, and sphingosine (Lloyd-Evans & Platt 2010). In the CNS, the GM2 and GM3 gangliosidoses are particularly prominent, while cholesterol accumulations is present, albeit to a lesser degree than what is observed in the peripheral tissues (Zervas *et al.* 2001).

Because of this defect in cholesterol trafficking, re-esterification of lipoprotein-derived cholesterol by ACAT is reduced (Lin *et al.* 2003, Pentchev *et al.* 1985). In addition, cholesterol levels in the plasma membrane of NPC-deficient cells may be low, even though total cellular cholesterol is increased (Sokol *et al.* 1988). Clinical manifestations of NPC disease include severe neuronal degeneration, hepatosplenomegaly, and effects in other organs (Patterson *et al.* 2001).

## Possible mechanism for NPC1 and NPC2 role in cholesterol efflux

NPC2 has been shown to transfer cholesterol *in vitro* between model membranes (Cheruku *et al.* 2006) and from membranes to NPC1-N-terminal domain (NPC1(NTD)) (Infante *et al.* 2008b). The crystal structure of NPC2 was solved in both the apo form (Friedland *et al.* 2003) and with cholesterol sulfate bound (Xu *et al.* 2007). From these structures it appears that there are no major conformational changes outside of the binding pocket of NPC2 associated with ligand binding. The structure of the NPC2/cholesterol sulfate complex has revealed that the ligand was positioned in the binding cavity with the iso-octyl side chain facing inwards and the sulfate moiety facing outwards to the solvent. Assuming that

cholesterol sulfate accurately represents cholesterol in this binding interaction, this orientation presents a topological problem for cholesterol extraction from and delivery to lipid membranes since cholesterol is positioned with the  $\beta$ -OH facing towards the hydrophilic solvent and with the iso-octyl side chain facing into the hydrophobic bilayer (Nakatani *et al.* 1996). Consequently, for NPC2 to bind cholesterol directly from or deliver to membranes in the orientation observed for cholesterol sulfate in the crystal structure, cholesterol would need to reverse its orientation as it transfers to or from the bilayer for proper positioning in the binding pocket of NPC2.

Alternatively, if NPC2 acts to transfer cholesterol from a protein donor or to a protein acceptor, which binds cholesterol in a complementarily reverse orientation (*i.e.*, with the iso-octyl side chain facing towards the solvent and with the  $\beta$ -OH facing inwards to a binding pocket) then no flipping of cholesterol would be required and NPC2 could shuttle cholesterol within the intralysosomal lumen and thus facilitate its efflux from that compartment. Precisely this complementary orientation has been observed in the recently elucidated crystal structure of the NPC1(NTD) (Kwon *et al.* 2009) and is supported by substantial biochemical evidence (Infante *et al.* 2008a). In one possible model of NPC1/2 function, upon cholesteryl ester hydrolysis by LAL, in the peripheral tissues, liberated cholesterol is transferred directly from LAL to NPC2 and then from NPC2 to NPC1(NTD) and then out of the lysosomal compartment (Figure 1). The structure of LAL is yet to be elucidated, however biochemical evidence suggests that LAL has significant preference for the length, number and the saturation of the fatty acid acyl chains, and only a weak preference for the nature of the esterified alcohol (Imanaka *et al.* 1984). This is consistent with LAL's ability to hydrolyze both cholesteryl esters and triacylglycerides. This preference points to the possibility that cholesteryl esters might be accommodated in the LAL binding pocket with an orientation that would facilitate the transfer of cholesterol to NPC2. Interestingly, both NPC2 (Cheruku *et al.* 2006) and LAL (Makino *et al.* 2006) activity *in vitro* are stimulated significantly by the presence of BMP, a specialized lipid of lysosomal membranes. BMP levels correlate with cholesterol levels in LE/LY (Chevallier *et al.* 2008), and BMP has been shown to accumulate in the LSOs of NPC cells (Kobayashi *et al.* 1999), possibly as a homeostatic response to increased cholesterol levels. It is unclear what role LAL might play in this model as it applies to the CNS as it is the *free* cholesterol/ApoE complex that mediates cholesterol intercellular transport in the CNS (Dietschy & Turley 2004).

While substantial biochemical characterization of the role of NPC1 and NPC2 in efflux of cholesterol from the LE/LY has been underway, precise elucidation of their molecular mechanism of action has been elusive. One major obstacle in establishing this mechanism has been inability of observing the proposed interaction of NPC1 and NPC2, although potential interaction surfaces of these proteins have been identified (Wang *et al.* 2010). Another obstacle has been, until the recent advances in the Brown and Goldstein group (Infante *et al.* 2008a), the availability of purified NPC1 protein for biochemical studies.

## Potential NPC disease therapeutics

### Substrate reduction strategies

Several strategies have been attempted for treatment of NPC disease. Substrate reduction approaches including statins, low-cholesterol diet and ezetimibe treatments, while reducing peripheral cholesterol burden failed in affecting NPC disease neuropathology and clinical outcomes (Patterson & Platt 2004, Liu *et al.* 2009). An alternative substrate reduction strategy for the remedy of the visceral phenotype would be to inhibit cholesterol production via the hydrolysis of cholesteryl esters by LAL. This approach has been shown to cause significant reduction in cholesterol storage in human NPC-defective cells (Rosenbaum *et al.*

2009), albeit accumulation of neutral lipids as the result of LAL inhibition might lead to Wolman disease-like complications at high levels of LAL inhibition and would not affect delivery of free cholesterol mediated by ApoE in the CNS. These substrate reduction approaches are not expected to correct insufficient steroidogenesis, which has been suggested to be an important part of NPC neuropathology (Griffin *et al.* 2004) or to affect delivery of free cholesterol mediated by ApoE in the CNS.

### Miglustat and Acid Sphingomyelinase treatments

Miglustat (N-butyl-deoxyojirimycin, Zavesca®) has been shown to reduce disease progression in NPC patients in clinical trials (Pineda *et al.* 2009, Wraith *et al.*, Patterson *et al.* 2007). Miglustat was originally developed as a therapeutic for Gaucher disease, a  $\beta$ -glucosidase deficiency (Pastores & Barnett 2003). Miglustat reduces glucosylceramide accumulation by inhibiting glucosylceramide synthase. Thus, miglustat leads to a reduction of glucosylceramide and an increase of ceramide levels. Chemical potential of sterols in membranes has been proposed to be a key regulatory factor in establishing cholesterol levels in different cellular compartments (Maxfield & Menon 2006, Mesmin & Maxfield 2009). Thus, alterations in membrane composition could lead to changes in the chemical activity coefficient of cholesterol and consequently changes in the distribution of cholesterol among membranes. Ceramide shares some chemical similarity with cholesterol in terms of having a relatively small polar headgroup (a hydroxyl in both cases) and it has been shown to displace cholesterol in lipid domains in model membranes (Megha & London 2004). Consequently, ceramide could alter the chemical activity coefficient of cholesterol in biological membranes, thus facilitating sterol efflux from the LSOs. Similarly, a reduction in sphingolipid (glucosylceramide itself and the complex gangliosides, for which it serves as a metabolic precursor) levels could lead to a similar increase in sterol chemical activity coefficient and increased cholesterol efflux.

Recent investigation of the interplay of sphingolipids and cholesterol in NPC disease has focused on acid sphingomyelinase (ASM) deficiency associated with NPC disease (Devlin *et al.* 2010). This study demonstrated that despite a normal ASM gene, NPC-deficient cells have much lower levels of ASM activity. Restoration of ASM activity levels via either transfection or exogenous addition of recombinant ASM reverted both cholesterol and BMP accumulation in both NPC1 and NPC2 mutant cells, and increased ASM activity restored normal protein trafficking (e.g., transferrin recycling). This beneficial outcome of the correction of a secondary defect indicates that NPC disease is a complex, multifaceted, disorder whose pathogenesis may include positive feedback loops that exacerbate the observed phenotype. This finding opens up new, previously unconsidered, pathways for treatment strategies, and it encourages the use of parallel therapeutic approaches that target multiple aspects of NPC pathology, and in particular the storage of glycosphingolipids associated with the neuropathology of NPC disease.

### Cyclodextrins

An important part of the NPC disease pathology involves insufficient delivery of cholesterol to mitochondria for steroid synthesis. Neurosteroid allopregnenolone (ALLO) has been shown deficient in NPC disease. Treatments of *npc1*<sup>-/-</sup> mice with ALLO, solubilized with 20 % hydroxypropyl- $\beta$ -cyclodextrin (HP $\beta$ CD), demonstrated significant improvements in murine viability and neuropathology (Griffin *et al.* 2004). However, subsequent studies by multiple investigators (Liu *et al.* 2009, Liu *et al.* 2010, Davidson *et al.* 2009) have shown that HP $\beta$ CD injections alone were responsible for most of the effect of HP $\beta$ CD+ALLO. These investigations have demonstrated that single injections into *npc1*<sup>-/-</sup> mice at P7 significantly reduced the NPC-phenotype in the brain, liver and other organs and resulted in increased viability. Single injections administered on later days were significantly less

effective. Repeated injections (Davidson *et al.* 2009, Ramirez *et al.* in press) were more effective in further prolongation of lifespan due to rapid elimination of cyclodextrins (CDs) in the urine (Liu *et al.* 2010). The decrease in efficacy in animals treated at later days could be attributed to at least two factors: closure of the blood-brain barrier and the progressive nature of NPC disease neuropathology.

CDs are membrane-impermeant cyclic oligosaccharides, which have been used extensively to modulate cholesterol and other lipids' composition of model and biological membranes (Zidovetzki & Levitan 2007) as well as to solubilize various pharmaceuticals (Davis & Brewster 2004). Despite their inability to cross membranes, CDs have been shown to be retained by cells (Kilsdonk *et al.* 1995). CDs can be delivered via pinocytosis to LE/LY, where they can replace the function of NPC1 and NPC2 proteins (Rosenbaum *et al.* 2010b) and promote cholesterol esterification by acetyl CoA:acyl transferase (Abi-Mosleh *et al.* 2009). The exact nature of CD's mechanism of action to replace NPC1 and NPC2 function in LE/LY is yet to be determined. One possibility would be that CDs could just solubilize cholesterol and thus promote its efflux via vesicular transport. Data with 70 kDa CD-dextran polymers (Rosenbaum *et al.* 2010b) that have been shown to be competent in reducing cholesterol storage and that colocalize with filipin staining of LSOs argue against this possibility since large dextran polymers have been shown to remain in LE/LY for extended periods of time (Mukherjee *et al.* 1997). Our preferred explanation for CDs action in this process is that they can act catalytically to shuttle cholesterol from LAL directly to the limiting membrane of the lysosome, bypassing the potential requirement for NPC2-NPC1 interaction (Figure 1). Once delivered to the luminal leaflet of the limiting membrane, cholesterol can then spontaneously flip to the cytosolic leaflet and be carried away to various destinations in the cell, via still poorly characterized transport processes (Maxfield & Menon 2006).

From the clinical treatment perspective, further optimization of the pharmacological properties of CD-based NPC therapeutics would be necessary to enhance CD delivery to the lysosomal compartment, enhance transcytosis across the blood-brain barrier, and decrease CD elimination through urine.

### Chemical Chaperones

Proteostasis modulation with chemical chaperones has been proposed as a therapy for a series of folding diseases, including lysosomal storage disorders (Balch *et al.* 2008). Enhancement of folding of the I1061T mutant of NPC1 protein with non-specific chemical chaperones that caused better trafficking of NPC1 to LE/LY and reduced NPC phenotype has been recently demonstrated in cultured cells (Gelsthorpe *et al.* 2008). This finding encourages further development of patient-specific chemical chaperone therapy for NPC disease. Further work would be needed in identifying the specific mutations that would be susceptible to such treatment as well as other chemical chaperones that might be more specific to NPC disease.

### Summary

While there are still remaining questions about the precise molecular mechanism of NPC1 and NPC2-mediated cholesterol efflux from LE/LY, our understanding of this critical step in cellular cholesterol homeostasis is rapidly improving. In addition, the rapid pace of the current development of novel therapies for NPC disease, less than two decades after the identification of the proteins involved, offers renewed hope for the NPC patients that suffer from this severe disorder. Moreover, recent reports of the role for NPC1 in Alzheimer's disease (Kosicek *et al.* 2010, Malnar *et al.* 2010, Kagedal *et al.* 2010) and human

immunodeficiency virus infection (Tang *et al.* 2009) make NPC disease research relevant to much broader audiences.

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## Abbreviations

<b>ALLO</b>	allopregnenolone
<b>ASM</b>	acid sphingomyelinase
<b>ApoE</b>	apolipoprotein E
<b>BMP</b>	bis(monoacylglycerol)phosphate
<b>CNS</b>	central nervous system
<b>CD</b>	cyclodextrin
<b>HP<math>\beta</math>CD</b>	hydroxypropyl- $\beta$ -cyclodextrin
<b>LAL</b>	lysosomal acid lipase
<b>LE/LY</b>	late endosomes/lysosomes
<b>LSO</b>	lysosomal storage organelle
<b>NPC</b>	Niemann-Pick type C disease
<b>NPC1(NTD)</b>	NPC1-N-terminal domain

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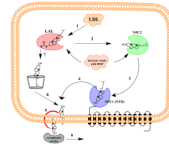
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**Figure 1.**

Model for NPC1, NPC2 and cyclodextrin action in cholesterol efflux from LE/LY. In normal cells, cholesteryl esters are hydrolyzed by LAL to produce free cholesterol and fatty acid (1), then free cholesterol is transferred to NPC2 (2), and subsequently to NPC1(NTD) (3). Free cholesterol is then delivered to the luminal leaflet of the limiting membrane of LE/LY (4), where it can spontaneously flip to the cytosolic side (5) and then be carried to its various destinations in the cell by cytoplasmic carriers (6). In the absence of functional NPC2 or NPC1, cyclodextrins can replace their functions in promoting cholesterol efflux (7, 8), bypassing steps 2–4. BMP stimulates the activities of LAL and NPC2. In the CNS, free cholesterol is shuttled from the glia to neurons via an ApoE complex, and no plasma LDL is believed to permeate the blood-brain barrier. Thus, all the CNS cholesterol is supplied through endogenous synthesis.