Cyclodextrin Overcomes Deficient Lysosome-to-Endoplasmic Reticulum Transport of Cholesterol in Niemann-Pick type C Cells.

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Abstract: A handoff model has been proposed to explain the egress from lysosomes of cholesterol derived from receptor-mediated endocytosis of LDL. Cholesterol is first bound by soluble Niemann-Pick C2 (NPC2) protein, which hands off the cholesterol to the N-terminal domain of membrane-bound NPC1. Cells lacking NPC1 or NPC2 accumulate LDL-derived cholesterol in lysosomes and fail to deliver LDL cholesterol to the endoplasmic reticulum (ER) for esterification by acyl-CoA acyltransferase (ACAT) and for inhibition of sterol regulatory element-binding protein cleavage. Here, we support this model by showing that the cholesterol transport defect in NPC1 mutant cells is restricted to lysosomal export. Other cholesterol transport pathways appear normal, including the movement of cholesterol from the plasma membrane to the ER after treatment of cells with 25-hydroxycholesterol or sphingomyelinase. The NPC1 or NPC2 block in cholesterol delivery to the ER can be overcome by 2-hydroxypropyl-beta-cyclodextrin, which leads to a marked increase in ACAT-mediated cholesterol esterification. The buildup of cholesteryl esters in the cytosol is expected to be much less toxic than the buildup of free cholesterol in the lysosomes of patients with mutations in NPC1 or NPC2.

Defect in Niemann Pick Type C impairs *Salmonella* infection

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The genetics of *Salmonella* and its regulation of cholesterol dynamics after intracellular infection provide powerful tools to identify host cell proteins that regulate late-endosomal, cholesterol trafficking and accumulation in cells. Niemann Pick Type C (NPC) disease cells accumulate cholesterol but the effector proteins underlying this trafficking defect remain poorly understood. When cultured Chinese Hamster Ovary cells truncated in the NPC gene were infected, bacterial proliferation was severely limited. We now show that *in vitro* grown fibroblasts derived from NPC patient are also refractory to *Salmonella* infection, with primary defect in intracellular replication rather than invasion. Ongoing studies are focused on (i) analyzing NPC patient fibroblasts for the function of a host protein PLEKHM2 known to be critical for intracellular cholesterol accumulation by *Salmonella,* and (ii) establishing whether NPC defect confers resistance to infection in *in vivo* models of disease, and thereby perpetuate disease genes in mammals.

Clinical Experience with Intravenous Infusions of Hydroxy-Propyl-Beta-Cyclodextrin in Identical Twin Patients with Niemann-Pick Type C Disease under Individual Investigator New Drug Exemptions

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Based upon animal studies of Niemann-Pick type C (NPC) disease demonstrating significant prolongation of life expectancy following the administration of hydroxy-propyl-beta-cyclodextrin (HPBCD), parents began pursuing treatment options with cyclodextrin for their children afflicted with NPC. In November 2008, individual investigator new drug exemptions (INDs) were filed with the FDA to provide intravenous infusions of HPBCD in these children. Following negotiations with FDA on the treatment protocol, and after obtaining institutional review board approval, twin children afflicted with NPC were surgically implanted with MediPort catheters. In April, 2009, the twins began receiving intravenous (IV) infusions of HPBCD. Initially, the patients received 4 days continuous IV infusions at 80 mg/kg/day. Subsequently, doses were titrated to a level of 2,500 mg/kg/day administered twice per week in 8-hour infusions. All infusions were performed in a pediatric subspecialty clinic at Renown Regional Medical Center in Reno, NV. Frequent safety monitoring, clinical labs, and biomarker samples were obtained throughout the more than 1 year of treatment.

Overall, the IV infusions of HPBCD were well tolerated. No infusion or delayed toxicities were observed. In fact, no significant changes were seen in vital signs or clinical labs. Of interest, and in contrast to findings in animal studies, the 2x elevations in baseline AST were not changed following HPBCD infusions. Oxysterol biomarkers, however, appear to have been reduced during the course of IV infusions. Pulmonary and auditory testing failed to show any deficits following nearly 1 year of HPBCD at the dose levels administered.

Although the parents believe the children are obtaining some therapeutic benefit from the IV infusions of HPBCD, the children continue to decline as evidenced by PET imaging and neurological assessment. Because recent findings suggest limited or no blood-brain barrier penetration of HPBCD following systemic administration, and direct central injection appears promising in animal studies, we are pursuing an intrathecal route of administration under the existing individual INDs. An update on the clinical plan and status of this approach will be presented.

A rapid neuromuscular and behavioural score for quantitative assessment of disease progression in a murine model of Niemann Pick Type C Disease.

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Niemann Pick Disease Type C (NP-C Disease) is a fatal, neurodegenerative, lysosomal storage disorder. It is a rare disease with broad clinical spectrum whose pathophysiology is poorly understood. Recent studies on emerging disease severity scales derived from patient natural history studies and clinical records suggest linear disease progression, independent of the age of onset. Here we adapt a quantitative, rapid murine, neuromuscular and behavioral scale to assess NP-C disease as manifested in the mouse model BALB/cNctr-$Npc1^{m1N}$/J, which carries a mutated copy of *npc1* resulting in a premature truncation.   Using this method a single mouse can be completely assessed within 3 minutes, enabling the operator to follow the evolution of disease in large numbers of animals. The resulting data suggests that, like in humans, disease progression is age-independent but linear, strongly reinforcing the model that symptomatic disease may be triggered by a 'threshold' state that varies on the basis of metabolism or other parameters heterogeneously achieved in genetically identical animals.   Disease progression can also be correlated with gene expression to identify aberrant pathways as targets of therapies. Hence the score provides a tool for initiation of treatment after the onset of cerebral disease (thus emulating the situation in the field) and by which an adjuvant therapy can be rapidly assessed.

Treatment of Niemann-Pick Type C Disease with Cyclodextrin

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Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disorder characterized by widespread intracellular accumulation of unesterified cholesterol and glycosphingolipids (GSLs). While there is no cure for NPC disease, there have been recent advances in the treatment of this disorder utilizing the compound 2-hydroxypropyl-beta-cyclodextrin (HPBCD). We and others have shown significant disease amelioration by administration of HPBCD to NPC1 mice yet many key questions as to how it provides this benefit remain to be answered. For example, is the ameliorating effect of HPBCD unique to NPC disease and if so, does HPBCD require the presence of at least one functional protein, either NPC1 or NPC2? Secondly, will different cyclodextrins (CD), such as alpha- and gamma-CDs or various derivatives of beta-CD, provide benefit similar to or better than HPBCD?

We have treated several murine models of other lysosomal diseases with HPBCD and results indicate that HPBCD does not ameliorate GSL or cholesterol storage present in these disorders. Thus, the beneficial effect of HPBCD may be unique to NPC disease. To determine whether at least one of the NPC proteins is necessary for HPBCD's effect, we generated NPC1/NPC2 double knock-out mice in house and treated them with HPBCD. The mice were administered HPBCD starting at postnatal day (P) 7 and continuing every other day until sacrifice at either P21 or P43. Tissues analyzed immunohistochemically and biochemically revealed that treated double knock-out mice showed benefit comparable to treated NPC1 mice, the more severe of the two individual phenotypes. Thus, even in the absence of both NPC1 and NPC2 proteins, HPBCD is able to reduce intraneuronal storage of cholesterol and GSLs.

While HPBCD has been shown to prevent and reduce cholesterol and GSL storage in NPC disease, it is possible that different types (alpha- or gamma-CD) or derivatives of CDs are more effective. To test this idea, we performed a series of short *in vivo* studies using several different CDs. NPC1 mice were administered CD from P7 to P21 every other day and sacrificed at P22. Immunohistochemical and biochemical analysis showed the following to be most effective to least effective in prevention of storage in NPC disease: HPBCD, methyl-beta-CD > sulfobutylether-gamma-CD > sulfobutylether-beta-CD > sulfobutylether-alpha-CD, 2-hydroxypropyl-alpha-CD. These results suggest that treatment efficacy, which may be influenced by the affinity of a specific CD for cholesterol and/or GSLs, depends not only on the type of CD but also on its chemical modifications.

Chemistry Core: Collaborative Drug Discovery Strategies for Niemann-Pick Type C Disease Therapeutics

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Abstract
    The Wiest and Helquist laboratories, specializing in computational chemistry and drug synthesis, are supported by the APMRF to provide a Chemistry Core for collaboration with NPC researchers in other areas of specialization to pursue the development of small molecule drugs to treat this disease. Prior work of other investigators has led to investigations of several agents, most prominent of which have been Miglustat, cyclodextrins, allopregnanolone, and oxysterols. However, a clear need exists for the continued development on an ongoing basis of compounds having improved efficacy.
    To establish a multifaceted strategy to investigate several potential drug classes and therapeutic targets, we have formed collaborations with many other laboratories, including the Maxfield lab at Cornell, the Liscum lab at Tufts, the Sturley lab at Columbia, the Ory lab at Washington University, the Repa laboratory at the University of Texas Southwestern Medical Center, and the D'Souza-Schorey and Haldar labs at Notre Dame. In the majority of these cases, compound screening and other preliminary studies in the labs of our collaborators have provided us with hits that have required computational models for understanding drug function, design of compounds having improved therapeutic properties, scaled-up syntheses for producing multi-gram quantities not available commercially, development of improved synthetic methods, and synthesis of new analogues. The resulting compounds have been provided back to our collaborators for more in-depth activity studies.
    Our labs have studied several classes of compounds, some of which have known cellular targets and mechanisms of action and others for which function must still be determined. These classes include histone deacetylase (HDAC) inhibitors (Maxfield, Repa, Ory, D'Souza-Schorey, and Haldar labs), new cyclodextrin derivatives (Maxfield lab), pyrrolinones and triazines that reduce lysomal cholesterol accumulation (Maxfield lab), a ceramide transport inhibitor (Sturley lab), lysosomal acid lipase inhibitors (Maxfield lab), and nitrovin (difurazone) having an unknown cellular mechanism (Liscum lab).
    In our presentation, we will place emphasis on computational modeling, compound selection, and chemical synthesis efforts employed in pursuing the leads from our collaborators. The details of follow-up activity studies of the resulting compounds, especially the HDAC inhibitors and cyclodextrins, by our collaborators will be provided in their separate presentations.
    The computational and drug synthesis capabilities of the Chemistry Core continue to be available to additional NPC investigators having a need for such collaborations.

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Temporal and spatial rescue of Niemann Pick type C disease

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Niemann-Pick C disease is a lysosomal storage disorder caused by genetic defects in the *NPC1* gene. As a result of loss of NPC1 function transport of cellular organelles is perturbed and large quantities of intracellular cholesterol accumulate. These defects lead predominantly to liver and brain degeneration. To determine the relevance of different cell types to the etiology and pathology of the disease, we have engineered mice in which production of a tagged NPC1 protein can be spatially and temporally controlled. A transgene encoding NPC1-YFP under the control of the Tet regulatory system can be expressed in specific cell types and activated or inactivated using doxycycline. The NPC1-YFP protein used is traceable and can restore function to $Npc1^{-/-}$ mutant cells in vivo. Using different mouse driver lines we have produced NPC1-YFP protein specifically in hepatocytes in the liver and in either astrocytes or neurons in the brain. Expression of the transgene at high levels in astrocytes did not rescue neurodegeneration, but expression at low levels in Purkinje neurons, or in other neurons, did. On this basis it seems that NPC pathology in the brain is mainly a neuron-autonomous process. We observe an inverse correlation between neuronal rescue and inflammation, measured as microglia activity. Rescue of $Npc1^{-/-}$ mutant mice with NPC1-YFP in hepatocytes also reduced inflammation, as measured by macrophage activity. These findings suggest that microglia and macrophages react to neuronal or hepatocyte dysfunction and are not harmful to healthy cells. By using doxycycline to control when NPC1-YFP is produced during the course of the disease, we aim to determine at what stages the disease is reversible, an important issue considering little is known about when to treat the disease. We have tested production of NPC1-YFP in liver at different stages of disease progression to determine whether the disease is reversible at later stages of the disorder. Inducing production of NPC1-YFP for a week, after the disease has progressed for seven weeks, significantly improved liver function, showing that NPC liver pathology is reversible. We are now continuing to perform this kind of experiment with our neuron rescue mouse lines.

Cholesterol Binding to N-terminal Domain of NPC1 is Essential for NPC1 Function in Mice

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Niemann-Pick type C (NPC) disease is an inheritable disorder caused by loss-of-function mutations in either *NPC1* or *NPC2* gene and characterized by lysosomal accumulation of cholesterol and other lipids. Evidence indicates that the primary cause is a failure of LDL-derived cholesterol to exit the lysosomes, which secondarily causes the buildup of other lipids. The molecular mechanism by which mutations in either *NPC1* or *NPC2* cause the same defect in this exit process remains poorly understood. Recently, we have shown that the egress of cholesterol from lysosomes requires a hydrophobic handoff of cholesterol from soluble NPC2 to the N-terminal domain (NTD) of membrane-bound NPC1. Using alanine-scanning mutagenesis, we further identified the amino acid residues on NPC2 and NPC1 that are essential for this cholesterol binding and transfer process. As a first step to elucidate the *in vivo* role of this hydrophobic handoff, we generated a line of NPC1 knock-in mice ($NPC1^{P202A;F203A}$) in which two point mutations were introduced into the NTD of NPC1. These mutations abolish cholesterol binding to NPC1(NTD) *in vitro* and eliminate NPC1 function in cultured cells. The mutant mice accumulated cholesterol in multiple tissues and exhibited the same lethal phenotype (with a mean lifespan of 80 days) as mice lacking either NPC1 (npc^{nih}) or NPC2 ($NPC2^{-/-}$). These data confirm that the cholesterol binding site in NPC1(NTD) is essential for NPC1 function in live animals. We are in the process of generating a line of *NPC2* knock-in mice harboring a mutant NPC2 ($NPC2^{I81D}$) that binds cholesterol normally, but cannot transfer its bound cholesterol to NPC1. If these mice develop NPC disease, the data will provide strong support for the hydrophobic handoff model *in vivo*.

Tissue Culture Models for Assessing NPC Therapies

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We have developed microscopy tools and biochemical assays to analyze the effects of various treatments on human fibroblasts obtained from patients with Niemann Pick C1 or C2 mutations. The goal of the work is to identify and test potential therapies for NPC disease. This information should lead to optimization of therapeutic strategies to be tested in animal models.

We recently reported that delivery of cyclodextrins to late endosomes and lysosomes is necessary for their effects in reducing cholesterol storage in NPC cells (1). We are testing ways to optimize the delivery of cyclodextrins to late endosomes and lysosomes. One strategy is to couple cyclodextrins to proteins that bind to receptors. Preliminary data suggest that α2-macroglobulin (α2M) may be a good candidate. The α2M binds to a receptor called LRP, which is expressed in many cell types and has also been shown to facilitate transport across the blood brain barrier. In many cell types α2M is delivered to late endosomes and lysosomes, where it is degraded (2). In collaboration with the Helquist laboratory, we are also optimizing cyclodextrin conjugates to polymers.

We are examining the effects of histone deacetylase (HDAC) inhibitors (HDACi) on NPC cells. We have found that certain HDACi can reduce the cholesterol storage in human NPC1-mutant fibroblasts. Data will be presented on the dose and time dependence of these effects as well as toxicity studies. We are also examining the mechanism of action of these HDACi, and the results of these mechanistic studies will be presented.

We are also developing methods to test therapies on several specific NPC1 mutations in collaboration with Bill Balch, and preliminary data on this work will also be presented.

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Identification of Surface Residues on Niemann-Pick C2 Essential for Hydrophobic Handoff of Cholesterol to NPC1 in Lysosomes

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Abstract: Water-soluble Niemann-Pick C2 (NPC2) and membrane-bound NPC1 are cholesterol-binding lysosomal proteins required for export of lipoprotein- derived cholesterol from lysosomes. The binding site in NPC1 is located in its N-terminal domain (NTD), which projects into the lysosomal lumen. Here we perform alanine-scanning mutagenesis to identify residues in NPC2 that are essential for transfer of cholesterol to NPC1(NTD). Transfer requires three residues that form a patch on the surface of NPC2. We previously identified a patch of residues on the surface of NPC1(NTD) that are required for transfer. We present a model in which these two surface patches on NPC2 and NPC1(NTD) interact, thereby opening an entry pore on NPC1(NTD) and allowing cholesterol to transfer without passing through the water phase. We refer to this transfer as a hydrophobic handoff and hypothesize that this handoff is essential for cholesterol export from lysosomes.

Pulmonary Function and Pathology in Treated and Untreated *Npc1-/-* Mice

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Lung pathology in the various diseases which were included under the rubric of Niemann-Pick in the 1960s was characterized only as micronodularity on x-ray. More modern studies in NPC2 have characterized it as consisting of alveolar proteinosis. We have studied the pulmonary disease in the $Npc1^{-/-}$ mouse. On histology, we find large numbers of alveolar foamy macrophages but no alveolar proteinosis. Using the flexiVent small animal ventilator (SCIREQ, Inc.), we find lung weight as % of body weight, inspiratory capacity (both influenced by the decreasing absolute weight of the near terminal mice), elastance and hysterisivity to be increased while resistance was not changed. Cholesterol measurements show a doubling of lung cholesterol levels. In a small number of $Npc1^{-/-}$ mice treated with cyclodextrin starting at 7 days, there is a trend towards heavier lungs and increased inspiratory capacity compared to the untreated mice. There is no increase in cholesterol per protein but, with the heavier lungs, there would be a larger absolute amount. We have started to investigate these parameters in the $NPC1^{nmf164/nmf/164}$ mice which have a longer survival time and, with their point mutation, are a better model of the human disease.

Functional analysis of NPC1 protein

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The NPC1 protein is comprised of 1278 amino acids with an apparent mass of 170–190 kD. The protein has 13 transmembrane domains, three large lumenal domains, and a cytoplasmic tail. To avoid the challenges of working with an extremely hydrophobic protein, we have isolated lumenal domain 2 in a soluble form by replacing the adjacent transmembrane domains with anti-parallel coiled coil sequences. The NPC1 domain 2 protein can be purified to biochemical homogeneity from the medium of cultured HEK293F cells grown in the absence of serum with reproducible yields of 100µg from a 60 ml culture. The purified protein has been used to test for direct interaction between NPC1 domain 2 and purified, bovine NPC2. Using surface plasmon resonance, preliminary experiments reveal direct interaction between immobilized NPC2 and purified NPC1 domain 2 that has been flowed across the chip surface. In addition, both purified NPC1 domain 2 and intact, endogenous, solubilized NPC1 protein have been used for affinity purification experiments to identify binding partners. Domain 2 shows specific interaction with two polypeptides of 17 and 19kD; intact NPC1 shows specific binding to a protein on 120kD. Mass spectrometry is being used to determine the precise identity of these binding partners. Our hope is that partner identification will add important information regarding NPC1 function. Finally, with the goal of determining the structure of NPC1 domain 2, we attempted to generate this domain in a less highly glycosylated form. Five constructs were expressed in HEK293F cells: Domain 2 missing any one of the first three glycosylation sites, a construct missing both sites 1 and 2, and a construct missing sites 1, 2 and 3. All of these were capable of expression in cells at similar levels, however all displayed decreased efficiency of secretion in relation to a native construct. These experiments confirm that NPC1 domain 2 glycosylation sites 1, 2 and 3 are indeed recognized by the glycosylation machinery in HEK293F cells, and oligosaccharide addition aids in the efficiency with which this protein is transported by the secretory pathway machinery.

Development of Surrogate Markers for
Niemann-Pick Disease, type C

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Introduction: The heterogeneous clinical nature and variable age of onset in Niemann-Pick Disease, type C (NPC) complicates the development of biomarkers to characterize disease burden and to evaluate efficacy of prospective therapeutics. Biomarkers in human cerebrospinal fluid (CSF) have the potential to both provide valuable insight into the neurologic pathology of NPC and to serve as surrogate markers in future therapeutic trials.

Methods: Between August 2006 and August 2010 we enrolled 52 NPC1 patients into a longitudinal observational study. Blood, urine, CSF and fibroblast cell lines have been collected. Multiple intramural and extramural collaborations have been established to analyze these samples. The NIH Severity Scale has also been implemented in the analysis of these data to correlate results to disease status. Murine studies are being used to complement investigations using human biomaterials.

Results: Several studies have yielded promising results. We have developed a quantitative assay to determine CSF calbindin-D levels. Calbindin-D is a marker for Purkinje cell damage. Although no correlation with disease status was observed, we did observe a trend ($p=0.08$) toward decreased calbindin-D levels in miglustat treated patients. Intriguingly in a subset of five patients on whom we have pre and post treatment values, calbindin-D levels decrease dramatically after treatment. An additional collaboration allowed us to analyze CSF from NPC patients and pediatric controls for Aβ, total-tau (T-tau) and phospho-tau (P-tau). T-tau and Aβ release is increased in NPC, and normal sAPP-β levels in patients suggest augmented activity of γ-secretase rather than β-secretase. These findings indicate that CSF T-tau may be useful as a biomarker in NPC treatment. Similar to calbindin-D, CSF T-tau levels appear to respond to miglustat therapy. CSF profiling using MALDI-TOF clearly distinguishes NPC from age matched control CSF. Future work will focus on identifying the peptides that contribute to these differences.

Summary: Both targeted and untargeted analysis of CSF from NPC1 one patients has identified significant differences NPC1 and control subjects. Initial analysis with both calbindin-D and T-tau suggest a response to miglustat therapy. Future work will focus on identifying additional CSF biomarkers and validating their potential use as surrogate markers in therapeutic trials.

Pulmonary Disease in the Niemann-Pick Type C Mouse

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Background: Niemann-Pick Type C (NPC) disease is an autosomal recessive lysosomal storage disorder characterized by a defect in cholesterol trafficking leading to an accumulation of unesterified cholesterol within the late endosomal/lysosomal compartment. In children, multiple case reports of severe pulmonary involvement suggest that lung disease is often under recognized in Niemann-Pick Type C disease.[1,2] Although the *npc1*^{-/-} mouse has been the most widely used animal model for NPC disease research, much of this work has centered on brain and liver disease. Therefore, the present studies were carried out to learn more about lung disease in the *npc1*^{-/-} mouse at various stages of development. **Aim:** (1) To characterize the pathogenesis of lung disease in the *npc1*^{-/-} mouse, and (2) to determine if serial weekly subcutaneous administration of 2-hydroxypropyl-β-cyclodextrin (CYCLO) ameliorates lung disease.
Methods: The following parameters were measured at specific stages of development ranging from weaning (19 days) to 70 days of age: lung weight, lung cholesterol synthesis and cholesterol concentration, and the relative level of expression of mRNA for various markers of inflammation. In addition, pulmonary function tests were measured in matching *npc1*^{+/+} and *npc1*^{-/-} mice using an unrestrained whole body plethysmograph chamber. This instrument measures a unit-less index of airway obstruction, known as enhanced pause (Penh). Lung histology was also assessed at 28, 49, and 70 days of age. **Results:** Relative lung weights were consistently higher in the *npc1*^{-/-} mice compared to their *npc1*^{+/+} counterparts at every age. Lung cholesterol concentration was also markedly elevated starting at 19 days of age, and remained so until the time of death. These findings correlated with the pulmonary function tests, which revealed a significantly greater level of airway obstruction in the *npc1*^{-/-} mice. Based on H & E staining, the lung showed notable accumulation of lipid laden macrophages within the alveoli of the *npc1*^{-/-} mice by 70 days of age. When *npc1*^{-/-} mice were treated with weekly subcutaneous injection of CYCLO starting at 7 days of age and studied at 49 days, the lung cholesterol concentration and cholesterol synthesis rates remained as elevated as in the untreated *npc1*^{-/-} animals. This is in sharp contrast to the liver and brain where both parameters were maintained at the levels observed in the *npc1*^{+/+} mice. The relative mRNA levels for inflammatory proteins, CD68, CD11c, and TNFα, were markedly elevated in the lung of the *npc1*^{-/-} mice but this inflammation did not respond to CYCLO treatment. **Conclusion:** Lung disease in the *npc1*^{-/-} mouse starts early in development and progresses with time. This finding supports a previous study looking at ultrastructural changes in the lung of the NPC1 mouse.[3] For this reason, it should be more widely recognized that children with NPC disease may also have a pulmonary component and should therefore be routinely screened with pulmonary function tests early on. Further studies are needed to determine whether aerosolized CYCLO delivered directly into the lungs can ameliorate pulmonary disease in the *npc1*^{-/-} mouse, and potentially, in children.

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Comparing the Molecular Mechanisms of Novel Therapies for NPC Disease

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Niemann Pick type C disease is a genetic disorder in which lipids (unesterified cholesterol, sphingomyelin, and glycolipids) accumulate in the late endosomes/lysosomes of cells throughout the body, resulting in liver, lung and neurologic symptoms. Our recent work has identified two agents that act in the $Npc1^{-/-}$ mouse model to enhance Purkinje cell survival, alter cholesterol dynamics in the central nervous system, and prolong lifespan: the LXR agonist T0901317 (oral dosing at 50 mg/kg body weight) and 2-hydroxypropyl-β-cyclodextrin (Cyclo, provided as single or repeated subcutaneous injections at 4000mg/kg body weight)[1,2]. During these studies we have identified gene expression "signatures" in CNS and peripheral tissues of $Npc^{-/-}$ mice treated with these agents to suggest that these drugs act to relieve lysosomal cholesterol accumulation.

As reported at the 2009 APMRF NPC Scientific Conference, a high-throughput screening effort spearheaded by the Wiest and Helquist labs at Notre Dame (Chemistry Core) and the Maxfield group (human NPC fibroblast filipin-based screening strategy) identified a number of novel compounds that appear to reduce lysosomal cholesterol accumulation in NPC fibroblasts. Our group has initiated collaborative studies on one class of these compounds, the histone deacetylase (HDAC) inhibitors.

Using a complement of cell-based approaches (primary neurons and macrophages) and whole-animal models (subcutaneous injection of 7 day-old $Npc1^{-/-}$ pups) studies are underway to evaluate these lead compounds. We will report on the effects of these agents on cholesterol balance, neurodegeneration, inflammation and lifespan. Importantly, we will compare and contrast the effects of these HDAC inhibitors against our previous readouts for Cyclo and LXR therapies.

[1] Repa, et al. 2007. Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. *J. Neurosci.* 27:14470-14480.
[2] Liu, et al. 2009. Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the $npc1^{-/-}$ mouse. *Proc. Natl. Acad. Sci. USA* 106: 2377-82.

Characterizing the role of TMEM97 in NPC disease

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Niemann-Pick Type C (NPC) disease is a genetic disorder in which defects in the lysosomal cholesterol transporters NPC1 or NPC2 cause accumulation of unesterified LDL-derived cholesterol in lysosomes. Recently, we have described the yet uncharacterized SREBP target gene TMEM97 as a putative regulator of NPC1 function (Bartz et al., 2009): Using a large-scale microscope-based screening approach we could demonstrate that silencing of TMEM97 with siRNAs reduces both, the uptake of LDL-cholesterol into cells as well as cholesterol levels in lysosomes. While in the presence of sterols TMEM97 localizes to the ER, a large fraction of the protein can be found in lysosomes and the plasma membrane when cellular cholesterol levels are low. Importantly, we could show that TMEM97 may co-localize and co-immunoprecipitate with NPC1 protein, suggesting a relevance for lysosomal cholesterol export. Here we have analyzed the role of TMEM97 in NPC-disease. Using several independent approaches we show that cellular levels of TMEM97 strictly depend on the levels of NPC1 protein. Moreover, our new data indicate an important role for TMEM97 on NPC1 levels and function and introduce TMEM97 as a potential new target for future NPC therapies.

Cyclodextrin uses an NPC2-like mechanism to transport cholesterol in Niemann Pick C disease

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Cholesterol accumulation in the late endo/lysosomal (LE/LY) compartment is the cellular hallmark of Niemann-Pick C (NPC) disease. Cyclodextrin (CD) has been shown to induce the release of accumulated cholesterol from the LE/LY compartment in NPC1 and NPC2 deficient mice, resulting in diminished neuropathy and increased life span, however the molecular mechanisms by which CD restores the normal transport of cholesterol are not known. In the present study we used *in vitro* model systems and fluorescence spectroscopy to examine the regulation and mechanism of sterol transport by CD. Transfer of cholesterol from three different species of CD to the lysosomal cholesterol binding protein, NPC2, occurs very slowly via aqueous diffusion, indicating that direct interaction between NPC2 and CD do not occur. In contrast, transfer of the fluorescent cholesterol analogue dehydroergosterol (DHE) from CD to phospholipid membranes, or from membranes to CD, was found to occur rapidly and in direct proportion to acceptor membrane or CD concentrations, respectively. Moreover, CD dramatically increases the rate of sterol transfer between membranes, again in direct proportion to membrane concentration. These results suggest that CD utilizes a collisional mechanism to transport cholesterol between membranes, with transfer occurring during direct interaction between CD and the membrane bilayer. Such a mechanism is similar to what we previously reported found for NPC2. Unlike NPC2, however, no effects of the endo/lysosome-specific lipid lyso-bis phosphatidic acid, were found for sterol transfer in the CD studies. Marked enhancement of sterol transport by CD was also found in cultured *npc2*$^{-/-}$ fibroblasts, where addition of CD rapidly rescued the cholesterol accumulation phenotype, as assessed by filipin staining. Thus, the recent observations of CD efficacy in mouse models of NPC disease are likely the result of CD enhancement of cholesterol transport between membranes, with rapid sterol transfer occurring during direct CD-membrane interactions.

Trafficking of Human NPC1 Mutants Responsible for NPC Disease

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Niemann-Pick Type C (NPC) is a neurodegenerative disease caused by the accumulation of unesterified cholesterol and sphingolipids at the endosomal-lysosomal (E/L) compartment. About 95% of NPC disease is due to the defective function of Niemann-Pick Type C1 (NPC1) and remaining 5% is contributed by NPC2. ~240 disease-causing mutations in NPC1 have been reported in the clinic and they include both missense and nonsense mutants. The most prevalent NPC1 mutant, I1061T has been reported by Ory and colleagues (J. Biol. Chem. (2008) 283:8229) to be misfolded and to show a trafficking defect preceding delivery to the E/L compartment resulting in a cholesterol imbalance in the cell. The trafficking and localization of the other NPC1 mutants is poorly understood. To generate a more systems level overview of the origins of the disease etiology across the NPC patient population, we have now generated a collection of plasmids that each harbor one of ~120 distinct NPC1 mutations that are found in the different domains of NPC1. Each of these NPC1 mutant proteins were transiently expressed in NPC1-deficient (npc1-/-) cell lines, and their expression and trafficking patterns were analyzed by immunofluorescence and for acquisition of endoglycosidase H (endo H), a hallmark of exit from the endoplasmic reticulum (ER) and delivery to/transit through the Golgi to the E/L compartment. Whereas wild-type NPC1, as expected, shows efficient acquisition of endo H-resistance and trafficking to the E/L compartment, a large number of mutations in the N-terminal domain, the cysteine-rich domain and most mutations in the sterol-sensing domain (SSD) remain sensitive to endo H, suggesting that NPC is largely a trafficking disease reflecting loss of interaction with the proteostasis network that facilitates normal folding and function (Balch et al. (2008) Science 391: 916; Powers et al. (2009) Ann. Rev. Biochem 78: 959-991). These mutants could be misfolded in the endoplasmic reticulum (ER) and potentially prone to ER-associated degradation (ERAD) and/or lead to an aggregation phenotype triggering the unfolded response (UPR) and cell death. Similarly, human NPC fibroblasts showed defective in NPC1 trafficking and cholesterol efflux from E/L compartment. Studies are currently in progress to further characterize these NPC1 mutants based on their localization, and their function in cholesterol homeostasis by NPC1 proteomics approach. In addition, the role of small molecules and pharmacological chaperones will be addressed to assess their potential role in the correction NPC1 mutants exhibiting folding and trafficking defects, likely due to an energetically destabilized fold that cannot be recognized by the proteostasis network.

Evaluating the Acute Effects of Cyclodextrin in *Npc1*^{-/-} Mice.

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Niemann-Pick Type C disease (NPC) is a rare disorder in which there is a defect in the intracellular transport of cholesterol from the late endosome/early lysosome that results in lipid accumulation. Patients typically suffer from hepatic steatosis, splenomegaly, pulmonary disease, and neurodegeneration, which results in premature death. Work by our group and others has shown that a single subcutaneous injection of 2-hydroxypropyl-β-cyclodextrin (Cyclo) at 4000mg/kg body weight in 7-day old *Npc1*^{-/-} mice results in decreased hepatic and neuronal inflammation, delayed neurodegeneration, and ultimately prolonged lifespan[1]. Studies performed 24h after a single Cyclo injection to the 7 or 49 day old *Npc1*^{-/-} mouse have shown dramatic changes in sterol balance: a decrease in cholesterol synthesis; an increase in the ratio of esterified/unesterified cholesterol; a down-regulation of SREBP2 and its target genes; and an-upregulation of LXR target genes[2]. Overall these data suggest that Cyclo is able to mitigate the release of the lysosomal pool of cholesterol from *Npc1*^{-/-} cells within 24h. A time-course experiment[2] using ^{14}C-labelled Cyclo in 49 day-old mice demonstrated that Cyclo is cleared from the plasma in 3 h and from the whole body in 6 h after a subcutaneous dose of 4000mg/kg.

A series of studies was performed to evaluate the acute effects of Cyclo (hours 1-24 after administration). The goals of these experiments were to determine: 1) whether a complex of Cyclo/cholesterol is evident in serum or urine; 2) how quickly gene expression changes are observed in liver and intestine to affect cholesterol and/or bile acid clearance from the *Npc1*^{-/-} mouse; and 3) to confirm the temporal gene expression changes in particular cell types (neurons, macrophages treated in culture) that may be masked by evaluating whole-tissue RNA content.

Results: Serum lipoprotein profiles were obtained 1,2,3,6, and 12h following injection of Cyclo in 49-day-old *Npc1*^{-/-} mice. A modest increase in HDL-cholesterol was observed at 6h. The enzymatic assay used to measure cholesterol in serum fractions did not reveal additional peaks to suggest the presence of Cyclo/cholesterol. As this enzymatic assay may not detect cholesterol complexed with Cyclo, rigorous extraction of fractions (and pre- and post-flow through) was performed and no additional cholesterol was observed. Urine was collected for 48 hours following injection and cholesterol content was very low and identical in concentration to saline-injected *Npc1*^{-/-} mice. These results suggest that Cyclo/cholesterol is not released from cells into the bloodstream. Hepatic RNA expression of CYP7A1, the rate-limiting enzyme of bile acid synthesis, was elevated by 6h after injection, while intestinal expression of IBAT, the transporter which reclaims bile acids, was reduced. These gene changes are consistent with the observed increase in bile acid excretion by *Npc1*^{-/-} mice following Cyclo injection[2]. Gene expression changes related to sterol homeostasis and inflammation in *Npc1*^{-/-} mouse tissues following acute Cyclo exposure were confirmed in primary cultured cells from our *Npc1*^{-/-} mice.

1 Liu, et al. 2009. Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the *npc1*^{-/-} mouse. *Proc. Natl. Acad. Sci. USA* 106: 2377-82.
2 Liu, et al. 2010. Cyclodextrin overcomes the transport defect in nearly every organ of the newborn or mature NPC1 mouse leading to excretion of the sequestered cholesterol as bile acid. *J. Lipid Res*. 51:933-44.

Magnetic Resonance Imaging of Mouse Models of NPC Disease

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Magnetic Resonance Imaging (MRI) is a non-invasive imaging modality commonly used for diagnosis and monitoring of progression and treatment response in clinical neurological diseases. As animal models of NPC disease have become available, MRI studies have been undertaken to characterize the changes seen in the brain throughout the animals' lifetimes.

We have studied / are studying several NPC disease model mice, including:

1) $Npc1^{-/-}$ in which there is no NPC1 protein expressed in the brain,
2) GFAP-Npc1^E, *Npc1-/-*, in which the NPC1 protein is expressed in astrocytes within the brain,
3) NSE- Npc1^H, *Npc1-/-*, in which the NPC1 protein is expressed exclusively in neuronal cells,
4) A double transgenic combining models 2) and 3), in which the NPC1 protein is expressed in both astrocytes and neuronal cell types, and
5) $Npc1^{nmf164}$, in which the *Npc1* gene has a point mutation, which more closely resembles common human forms of the disease as compared to the $Npc1^{-/-}$ mouse model.

T2-weighted anatomical imaging and quantitative T2 imaging studies have been undertaken to examine the different types of NPC disease mouse models. Preliminary analyses suggest differences in myelination as well as differences in ventricular characteristics and brain structure volumes between the different types of mice models. Further analyses are underway to quantify the differences in myelination as measured by T2 measurements and brain structure morphology over time.

Longitudinal Magnetic Resonance Spectroscopy and T2 Measurements in a Mouse Model of Niemann-Pick Type C Disease

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Abnormal myelination has been reported in a case study of clinical NPC disease using Diffusion Tensor Imaging (DTI). Dismyelination in a mouse model of NPC disease has been reported shortly after weaning at 23 days of age and quantified with DTI experiments, but required 3 hours of scan time. Magnetic resonance spectroscopy (MRS) has been used in studies of several mouse models of neurodegenerative diseases, and in clinical studies of Niemann-Pick Type C disease, but has not been reported in the NPC mouse model. In this work, a longitudinal study of T2 mapping and MRS measurements in a mouse model of NPC disease has been performed to examine T2 relaxation and brain metabolite levels as indicators of disease progression and response to therapy.

Age matched wild-type (WT) control mice and NPC disease model mice (NPC) were scanned at weekly intervals from 22-43 days of age, and again at 64 days of age. T2-weighted datasets were collected with a 2D radial fast spin-echo sequence and T2 maps were calculated from 21 coronal slices within the brain. A region of interest analysis was used to obtain T2 values from the white matter areas of the external capsule, corpus callosum, and cingulum, while avoiding inclusion of ventricular spaces. MRS datasets were collected with a point-resolved spectroscopy (PRESS) sequence. 3mm cubic voxels were placed in the cortex and cerebellum areas of the mice and spectra were analyzed by calculating the ratio of the metabolite peak signals to that of the unsuppressed water signal from the spectroscopic voxel.

Results from the T2 study are shown below in Fig. 1. T2 values in white matter differ significantly between the WT and NPC groups at all time points studied. T2 relaxation times in both NPC and WT mice decrease with age. Relative metabolite peak intensities are shown in Fig. 2 for the spectral peaks corresponding to the metabolites Choline (Cho), Creatine (Cre), N-Acetyl Aspartate (NAA), and lipid peaks at 1.3 and 0.9 ppm. None of the metabolite levels are significantly different between the two groups of mice. From these results, it is likely that quantitative T2 mapping could play a role in non-invasive evaluation of NPC disease and its response to therapy in preclinical animal studies while MRS measurements appear to lack sensitivity to the disease.
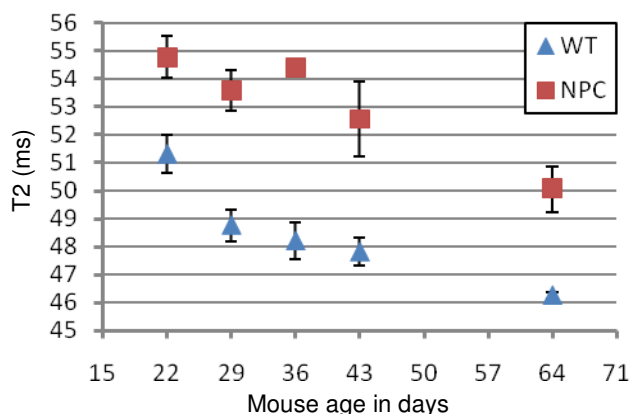

Figure 1. Values of T2 in white matter regions of groups of WT and NPC mice at 5 ages. T2 differences between the groups are significant at all timepoints (P<.005).
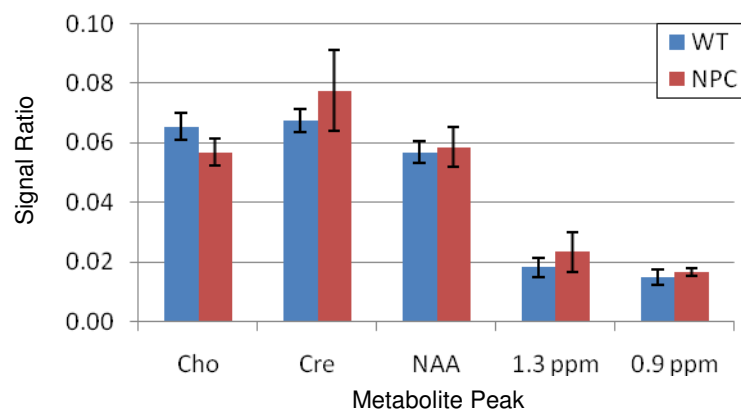
Figure 2. Relative metabolite measurements from 3mm cubic voxels in the cerebellum of 64 day old WT and NPC mice, differences between WT and NPC mice did not reach significance.

A Comparison of ERAD and Dynamic Membrane Models of NPC Disease.

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Mutations in NPC1 account for approximately 95% of all NPC cases, however the locations of specific mutations in the NPC1 gene fail to reveal crucial functional domains or activities. This aspect of the disease has bolstered support for the ERAD model which proposes that mutant NPC1 proteins do not fold during synthesis and are degraded. Parallels to the cystic fibrosis gene product CTFR suggest that this could be a common disease mechanism. To test the ERAD model for NPC1, we used a combination of live-cell imaging, expression of NPC1 and CTFRΔF508 proteins and MS/MS protein identification of NPC1 membrane fractions. To ensure that ER retention and degradation was detectable in our system, we first expressed CFTRΔF508 and observed substantial accumulation in reticular ER membranes. To visualize ER retention of NPC1 (wild-type and mutant), we treated with tunicamycin and observed clear ER accumulation. In untreated cells however, I1061T mutant NPC1 targeted to late endosomes/lysosomes (LE/Ls) rather than ER and colocalized with wild-type NPC1. This was not affected by MG132 treatment, an approach that blocks proteosome-mediated degradation. Finally, western blot analysis of mutant NPC1 from patient biopsy cell lines failed to reveal evidence of MG132-sensitive degradation. Because live-cell imaging of tagged-NPC1 constructs suggests a novel mechanism of cholesterol transport via tubular membranes, we identified molecular components required for membrane tubule formation and compared defects in these components to NPC1 mutants. Inactivating mutants of rab8a, EHD1 and SNX4 each blocked membrane tubule formation and induced cholesterol accumulation in LE/Ls similar to NPC1 mutant cells. These defects were also induced by microtubule depolymerization or shRNA of p150Glued, suggesting a requirement for membrane-microtubule interactions in tubule formation. Interestingly, treatment with rapamycin enhanced the degree of membrane tubulation in live-cell imaging and improved cholesterol efflux in I1061T mutant cells. Finally, we prepared NPC1 membranes from wild-type and I1061T mutant NPC cell lines and performed MS/MS analysis. Membranes from wild-type NPC1 cells contained LE/L-associated transmembrane proteins, GTPase-associated proteins, dynactin and cytoplasmic dynein. Membranes from a I1061T mutant cell contained a similar set of proteins. However, some proteins implicated in membrane tubule formation were lost from mutant samples, including dynein and dynactin. These studies suggest that NPC1 disease might not be a member of ERAD-associated diseases, supporting analysis of other mechanisms.

Treatment of Niemann-Pick type C Disease with Cyclodextrin – Case Report

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Introduction: Niemann Pick type C (NPC) is a rare autosomal recessive disorder caused by deficiency of protein NPC1 or NPC2, which are responsible for intracellular transport of non-esterified cholesterol and other sphingolipids, causing accumulation of these substances in the lysosomal/endosomal system, in various tissues, especially in the brain. It is a progressive disease, incurable so far, with death occurring within a few years after diagnosis. The clinical manifestations are not uniform, but the brain damage tends to occur during the course of the disease. The natural history of individuals with NPC follows a linear evolution over time, regardless of age of onset of symptoms. Until recently the treatment for NPC was restricted to drugs that were trying to control symptoms, such as anti-epileptics and antidepressants. In recent years some drugs have been developed or began to be used in these patients. Currently available options are N-acetylcysteine, curcumin and miglustat. Some drugs appear as therapeutic options, such as 2-hydroxypropyl-beta-cyclodextrin (Cyclodextrin) and imatinib.

Methods: A case report of two sisters with NPC, one aged 16 (Patient 1) and another with 12 years (Patient 2) after the use of miglustat and cyclodextrin (IV infusion, 1500mg/kg, twice a week, for eight months).

Results: Patient 1 had reduced the scale of severity after the use of miglustat, with improvement in dysphagia and vetrical supranuclear gaze palsy. After use of cyclodextrin was also improves memory, cognition, depression and cataplexy. The second patient did not present results with miglustat, but there was improvement in fine motor skills, ambulation, speech, incontinence and hallucinations after the use of cyclodextrin.

Discussion: The cyclodextrin is a starch that is component of various medications. Recent data suggest that the use of this drug in animal models with NPC reduces neuronal storage of cholesterol, with improvement in motor function and increased survival. After eight months, the cyclodextrin was effective in motor function, cataplexy, incontinence, memory, cognition, ambulation and psychiatric manifestations.

Conclusion: This is the first report of the use of cyclodextrin in humans with NPC. The reduction of severity scale changes the natural history curve of the disease, making cyclodextrin a promising therapeutic opinion for NPC patients. It is extremely important to start clinical trials to confirm the results found in this case report.

Intrathecal cyclodextrin therapy of feline Niemann-Pick type C disease.
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To more rigorously evaluate the mechanistic, pharmacologic, and toxicity issues associated with 2-hydroxypropyl-ß-cyclodextrin (HPßCD) therapy in NPC disease, we have utilized the spontaneous feline NPC disease model harboring a missense mutation in *NPC1* (pC955S), orthologous to the most common mutation in juvenile-onset patients. Disease progression in this model recapitulates both the neuropathological and biochemical abnormalities observed in this subset of human patients. Using the feline model of NPC disease, we have administered HPßCD directly into the subarachnoid space at the cerebellomedullary cistern and achieved highly encouraging results: the clinical neurological signs of disease were completely resolved up to at least 24 weeks of age (the median age when untreated cats die). Notably however, a completely unanticipated dose-related toxic effect of HPßCD on the auditory system was also observed. Moreover, parenterally administered high doses of HPßCD produced the same disease-modifying and ototoxic effects as that administered intrathecally leading us to hypothesize that sufficient concentrations of HPßCD crossed the blood brain barrier to achieve these CNS effects. We directly tested this hypothesis. We identified that parenterally administered HPßCD gains access to the CNS only when administered at doses greater than 1000 mg/kg. An average CSF concentration of 21.1 ug/ml was measured 60 minutes following administration of subcutaneous 8000 mg/kg. Plasma concentrations were also measured 60 minutes following administration and means were 546 ug/ml, 2570 ug/ml, and 3485 ug/ml in cats administered 1000 mg/kg, 4000 mg/kg, and 8000 mg/kg subcutaneously. These studies indicate that HPßCD can access the CNS when given parenterally at high concentrations, however, clearly studies are indicated to determine the maximum CSF concentration achieved following parenteral administration as well as the full pharmacokinetic profile of HPßCD in the CSF. Data on the pharmacokinetics following intrathecal administration will be presented. The studies will further determine if the protective and neurotoxic effects of HPßCD can be separated in a dose-dependent manner.

A major obstacle in clinical trials in human patients is the lack of validated surrogate markers of brain disease that can be monitored as secondary clinical endpoints. Our studies show that intrathecal administration of HPßCD ameliorates neurologic disease, but has no effect on hepatic or pulmonary disease. As such, we hypothesize that intrathecal treatment with HPßCD will allow us to effectively distinguish biochemical markers of NPC-related CNS disease from biomarkers of hepatic and pulmonary disease. Significant differences between affected and unaffected cats have been determined. Significant increases were found in plasma of NPC disease cats in 7-ketocholesterol and triol with triol having the best specificity and sensitivity. Increases in 7-beta-hydroxycholesterol and 27-hydroxycholesterol, both cholesterol oxidation products, were also found. Samples of CSF were collected from affected and normal cats and evaluated for small metabolites. Increases in CSF tryptophan and its metabolites; increases in arachidonate and lysolipids; and decreases in myoinositol, N-acetylneuraminate, N-acetylasparatate were identified.

Pathology and biochemistry of the brain from intrathecally-treated cats correlated with clinical neurologic findings. Remarkably, intrathecally-treated cats showed amelioration of neuronal swelling and axonal spheroid formation in many but not all brain regions, and preservation of Purkinje cell numbers. Brain lipid analysis showed markedly decreased gangliosides and sphingosine, and filipin staining showed decreased cholesterol. However, significant evidence of microgliosis and astrogliosis remained.

Modeling of Rare Diseases: From Networks to Drugs

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Abstract

Economic constraints are a key problem in the development of treatments for rare diseases. The typical cost of developing a drug to the point of FDA approval and marketing now approaches 1 billion US dollars. Even under the best of circumstances, the costs can be conservatively estimated to be 50-100 million US dollars, thus far exceeding the resources that are available for the treatment of the estimated 5000-7000 rare diseases.[1] The development of new chemical entities as drugs for the treatment of rare diseases will therefore be the rare exception rather than a promising general approach.

A more promising approach is the repurposing of already developed (and possibly FDA approved) compounds for the treatment of rare diseases. This approach leverages the investment already made to establish a safety profile and ADME/Tox properties in other studies (sometimes for other rare diseases) to make a treatment for another disease available at greatly reduced cost. This approach is not limited to FDA approved drugs because the side effects that prevented FDA approval for the treatment of a disease with alternative options might be acceptable in the case of a fatal rare disease where there are no other treatment options available. Depending on the stage of (pre)clinical development desired, this increases the number of potential compounds by at least 1-2 orders of magnitude beyond the ~1350 currently FDA approved compounds. Although the repurposing of existing compounds for the treatment of rare diseases has long been recognized as a promising approach and several studies in this direction have been proposed or executed, they are complicated by the lack of models for high-throughput assays and the lack of sufficient quantities of compounds to be tested.

Here, we describe the initial results of a different approach where we study a large set of rare diseases by linking the network of genetically caused rare diseases to pharmacologically well-studied small molecules that can be evaluated for their potential as treatments for rare diseases. This will be achieved by connecting the rare disease to the better studied human disease network[2] and a drug-target network.[3] Links between them can then used for searches in the target annotations of small-molecule databases such as the MDDR or Drugbank to identify candidates for experimental testing. If the structure of the target is known or can be determined by homology modeling, traditional methods of structure-based drug design will be used to validate the target assignment.

We have constructed a rare disease network based on the ORPHANET database containing 5781 annotated rare diseases and linked it to the network derived from the previously studied[2,3] OMIM-derived network. Information such as genetic origin, prevalence, age of onset or death were included where available. The resulting network clearly clusters into two very large clusters of rare diseases with multiple disease areas.
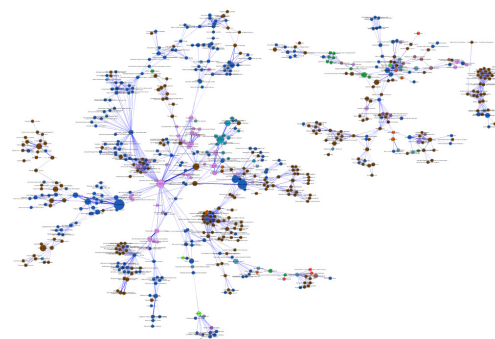
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A Role for Oxysterol Binding Protein-Related Protein 5 in Endosomal Cholesterol Trafficking

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Oxysterol binding protein (OSBP) and its related proteins (ORPs) constitute a large and evolutionarily conserved family of lipid binding proteins, which target organellar membranes to mediate sterol signaling and/or transport. Here we characterize a novel ORP, ORP5, and we find that ORP5 is a tail-anchored protein which localizes to the endoplasmic reticulum (ER). Knocking-down ORP5 causes cholesterol accumulation in the late endosomes and lysosomes, reminiscent of the cholesterol trafficking defect in Niemann Pick C (NPC) fibroblasts. Interestingly, cholesterol appears to accumulate in the limiting membranes of the endosomal compartments in ORP5 knockdown cells, whereas depletion of NPC1, or both ORP5 and NPC1 results in lumenal accumulation of cholesterol. Moreover, trans-Golgi resident proteins mis-localize to endosomal compartments upon ORP5 knockdown, which depends upon a functional NPC1. Our results suggest that ORP5 may cooperate with NPC1 to mediate the exit of cholesterol from endosomes/lysosomes.

Deconstructing Niemann-Pick type C neuropathology using conditional Npc1 knockout mice

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Our laboratory recently generated a conditional null allele of the mouse *Npc1* gene, in which *lox*P sites flank exon 9 (designated *Npc1 flox*). Here we used this system to study the timing and cell type that underlie neurodegeneration due to Npc1 deficiency. We initially sought to establish the extent to which *Npc1* deficiency during CNS development contributes to Niemann-Pick type C neuropathology. To accomplish this, we generated *Npc1 flox/-* compound heterozygotes expressing a tamoxifen regulated Cre recombinase under the control of the CMV promoter. Cre-mediated deletion of the floxed allele was induced by tamoxifen treatment of sexually mature adult mice at 6 weeks and resulted in widespread recombination throughout the brain. Our preliminary data show that global deletion of *Npc1* in adults leads to progressive weight loss, impaired balance beam performance, and early death in a time course similar to that resulting from germline deletion. These observations prompted us to determine the extent to which pathology arising in adult astrocytes contributes to neuropathology. We generated *Npc1 flox/-* mice expressing a tamoxifen regulated Cre from the astrocyte specific GFAP promoter. Cre activation was triggered by tamoxifen treatment of 6 week old mice, and resulted in efficient recombination in adult astrocytes in many brain regions, including the cortex, hippocampus, and cerebellum. These mice exhibit normal weight, motor function, and survival up to one year. In agreement with these *in vivo* findings, deletion of *Npc1* in primary astrocyte cultures does not impair survival despite the accumulation of unesterified cholesterol. Our findings demonstrate that deletion of *Npc1* in the adult is sufficient to cause disease and suggest that astrocytes are not a prime contributor to the Niemann-Pick type C phenotype.