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February 26, 2010

Tim Cote, MD  
Office Orphan Product Development  
Food and Drug Administration  
5600 Fishers Lane  
HFD-35, Room 6A-55  
Rockville, MD 20857

RE: Orphan Drug Designation Application

Dear Dr. Cote:

Enclosed please find an original, one hard copy, and an electronic copy (flash drive) of an application for Orphan Drug Designation for the use of Hydroxy-propyl-beta-cyclodextrin in the treatment of Niemann Pick Disease, Type C (NPC).

Thank you for your consideration of this application.

Sincerely,

Caroline Hastings, MD  
Director, Fellowship Program  
Department of Pediatric Hematology Oncology  
Children's Hospital & Research Center Oakland  
757 Fifty Second Street  
Oakland, CA 94609-1809

**Petition Requesting Orphan Designation  
for Use of Hydroxy-Propyl-Beta-Cyclodextrin in the  
Treatment of Niemann-Pick Disease Type C**

**February 26, 2010**

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**I. Statement Requesting Orphan Drug Designation for Use of Hydroxy-Propyl-Beta-Cyclodextrin in the Treatment of Niemann Pick Disease Type C**

*A sponsor's request for an orphan drug designation must contain a statement that the sponsor requests orphan drug designation for a rare disease or condition. The statement shall identify the drug and disease or condition with specificity.*

*21 C.F.R. § 316.20(b)(1).*

CHORI requests that Hydroxy-Propyl-Beta-Cyclodextrin ("HPBCD") receive an orphan drug designation for use in the treatment of patients with Niemann-Pick Disease Type C ("NPC"). There are fewer than 200,000 Niemann-Pick Disease Type C patients in the United States.

Based on current census data, and on prevalence data in the United States derived from modern screening techniques, there are estimated to be fewer than 2,000 patients in the United States with genetically-defined NPC. It is CHORI's understanding that because the total number of NPC patients is under 200,000, HPBCD will qualify for orphan designation for this indication.

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Caroline Hastings, M.D.

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Date

## II. Information about the Sponsor and Manufacturer

*A sponsor's request for an orphan drug designation must contain the name and address of the sponsor; the name of the sponsor's primary contact person and/or resident agent including title, address, and telephone number; the generic and trade name, if any, of the drug or drug product; and the name and address of the source of the drug if it is not manufactured by the sponsor. 21 C.F.R. § 316.20(b)(2).*

The following information addresses the requirements enumerated above:

**Sponsor:** Children's Hospital Oakland Research Institute (CHORI)

**Address:** 5700 Martin Luther King Jr. Way  
Oakland, CA 94609

**Contact:** Caroline Hastings, M.D.  
Director, Fellowship Program  
Department of Pediatric Hematology Oncology  
Children's Hospital & Research Center Oakland  
747 Fifty Second Street  
Oakland, CA 94609-1809

Telephone: (510) 428-3631

Facsimile: (510) 601-3916

E-mail: [chastings@mail.cho.org](mailto:chastings@mail.cho.org)

Generic and/or trade name: Hydroxy-Propyl-Beta-Cyclodextrin

Brand name: Trappsol™.

CHORI has obtained a source of endotoxin certified HPBCD (Trappsol™) from:

**CTD Holdings Inc.**  
27317 NW 78th Avenue  
High Springs, FL 32643  
Phone: 386-454-0887

Cross-Reference: DMF No. 74-145, contains additional information about the manufacturer of Hydroxy-Propyl-Beta-Cyclodextrin.

### III. Description of Niemann-Pick Disease Type C

*A sponsor's request for an orphan drug designation must contain a description of the rare disease or condition for which the drug is being or will be investigated, the proposed indication or indications for use of the drug, and the reasons why such therapy is needed. 21 C.F.R. § 316.20(b)(3).*

#### A. Description of Niemann-Pick Disease Type C

CHORI's Petition for Orphan Drug Designation for Hydroxy-Propyl-Beta-Cyclodextrin requests orphan designation for use of HPBCD in the treatment and management of Niemann-Pick Type Disease Type C (NPC) patients. NPC is an autosomal recessive lysosomal storage disease characterized by progressive neurodegeneration. More than 250 mutations of the NPC1 gene located on chromosome 18 have been identified. Although NPC has a highly variable phenotype, the classic presentation occurs in middle to late childhood with insidious onset of ataxia, vertical supranuclear gaze palsy, and dementia. Other features include dystonia, dysarthria and dysphagia eventually becoming disabling. Death typically occurs in the late second or third decade from aspiration pneumonia. Definitive diagnosis of NPC is based upon impaired cholesterol esterification and positive filipin staining in cultured fibroblasts. Although NPC differs in major respects from Alzheimer's disease, there are many parallels in the cellular pathology including neurofibrillary tangle formation, prominent lysosome system dysfunction, and influences of apolipoprotein E genotype. There are no FDA approved drugs for the treatment of NPC and current care is therefore palliative.

## 1. *Brief Overview of NPC*

Niemann-Pick Disease Type C, is a rare, fatal, genetic disease associated with impaired intracellular lipid trafficking and progressive neurological symptoms. An excellent overview of NPC is available on the internet.<sup>1</sup> NPC may present from infancy through adulthood but most commonly arises during middle-to-late childhood. Early-onset disease is usually recognized on detection of unexplained cholestatic disease. Most infantile-onset cases present with hepatosplenomegaly, thrombocytopenia, likely related to splenomegaly. Late infantile-onset cases present with hypotonia and developmental delay, followed by dystonia, ataxia, dysphagia, and dysarthria. Childhood-onset NPC typically presents as ataxia, cognitive impairment, and clumsiness. Impaired vertical saccadic eye movements are invariably present but are often overlooked. Gelastic cataplexy and epileptic seizures are also common; progressive dystonia, dysphagia, and dysarthria manifest as the disease advances. Disease progression and symptomatology and overall life expectancy appear to be strongly influenced by the age at symptom onset. Children with early-onset disease tend to experience rapid symptomatic decline, with death often occurring before the age of 5 years. Many patients with childhood-onset disease die before 20 years of age, although some survive into their 30s.”<sup>2</sup> Definitive diagnosis of NPC is based upon the demonstration of impaired LDL-cholesterol trafficking in cultured fibroblasts of patients by cytochemical visualization of accumulated free cholesterol after filipin staining.

## 2. *The NPC Gene*

Niemann-Pick Disease Type C is inherited in autosomal recessive fashion and is attributed to mutations in the genes NPC1 (located on chromosome 18q11-q12 and responsible for 95% of cases) and NPC2 (mapped to chromosome 14q24.3).<sup>3</sup> These gene mutations result in identical cellular and biochemical phenotypes consisting of severe impairment of intracellular lipid transport leading to the accumulation of unesterified cholesterol and other lipids in perinuclear lysosomes. More than 200 different mutations in these genes have been identified to date.

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<sup>1</sup> <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=npv>

<sup>2</sup> Patterson et.al. J. Child Neurol. Oct 12, 2009.

<sup>3</sup> Millat et al, Am. J. Hum. Genet, 2001 a,b

### 3. Need for Treatment in NPC Patients

Because no FDA approved drugs are available to treat NPC patients a great need exists for any therapy that may prove beneficial. Recently, the use of miglustat (Zavesca<sup>®</sup>) has shown promise in slowing the progression of NPC disease in children.<sup>2</sup> Miglustat is a small aminosugar molecule that reversibly inhibits glucosylceramide synthase and has shown beneficial effects in studies of murine and feline models of the disease.<sup>4</sup> Nevertheless, miglustat does not normalize the NPC phenotype, has significant side effect liability, and is extremely expensive due to its non-approved status.

## IV. Scientific Rationale for Use of Hydroxy-Propyl-Beta-Cyclodextrin in the Treatment of Niemann-Pick Disease Type C

*A sponsor's request for an orphan drug designation must contain a description of the drug and a discussion of the scientific rationale for the use of the drug for the rare disease or condition, including all data from non-clinical laboratory studies, clinical investigations, and other relevant data that are available to the sponsor, whether positive, negative, or inconclusive. The sponsor must include copies of pertinent unpublished and published papers. 21 C.F.R. § 316.20(b)(4).*

### A. Description of Hydroxy-Propyl-Beta-Cyclodextrin

- 1) Chemistry and Manufacturing
  - a) Chemistry

Cyclodextrin molecules are relatively large (molecular weight ranging from almost 1000 to > 2000 Da) with a large number of hydrogen donors and acceptors, and are consequently poorly absorbed through biological membranes. Cyclodextrins are non-reducing cyclic glucose oligosaccharides resulting from the cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19; CGTase) catalyzed degradation of starch. Their structures have been reviewed. There are three common cyclodextrins with 6, 7 or 8 D-glucopyranosyl residues ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrin respectively)

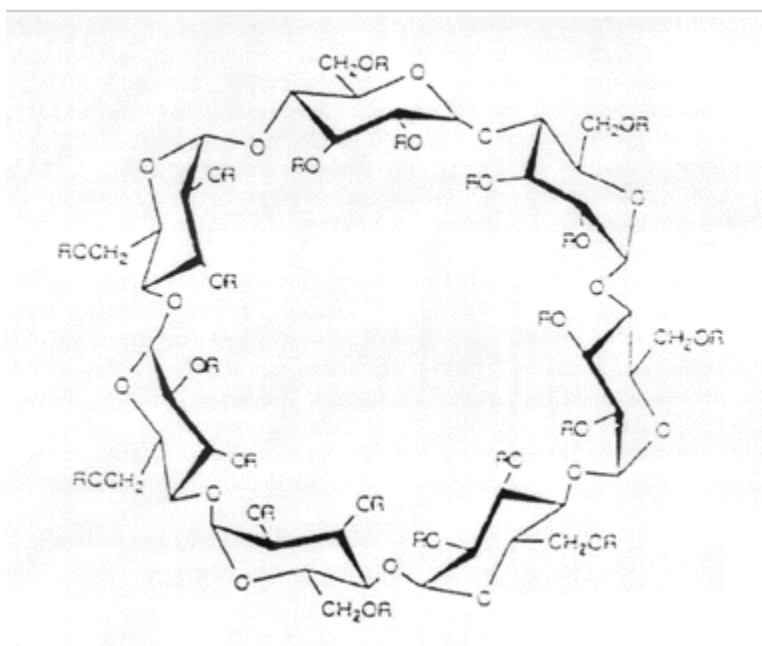
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<sup>4</sup> Zervas et al 2001

linked by  $\alpha$ -1,4 glycosidic bonds. The glucose residues have the  $^4C_1$  (chair) conformation. All three cyclodextrins have similar structures (that is, bond lengths and orientations) apart from the structural necessities of accommodating a different number of glucose residues.

Hydroxy-Propyl-Beta Cyclodextrin (HPCD) (cas#94035-02-6) is a partially substituted poly (hydroxypropyl) ether of beta cyclodextrin (BCD). The empirical formula is:  $(C_{42}H_{70-n}O_{35}) \cdot (C_3H_7O)_n$ . It contains not less than 10.0 percent and not more than 45.0 percent hydroxypropoxy(-OCH<sub>2</sub>CHOHCH<sub>3</sub>) groups. The structure is shown below where R represents either hydrogen or a hydroxypropoxy group.

R= CH<sub>2</sub>CH(OH)CH<sub>3</sub> or H



The solubility of HPBCD is quite high, exceeding 600 mg/ml. Viscosity is not an issue in concentrations below 55%.

#### b) Source

CHORI has obtained a source of Hydroxy-Propyl-Beta-Cyclodextrin as Trappsol® brand of endotoxin controlled HPBCD will be obtained from Cyclodextrin Technologies Development, Inc. 27317 NW 78<sup>th</sup>

Ave, High Springs, FL 32643 Telephone: (386)-454-0887. The product is referenced in Drug Masterfile 10772.

c) Human pharmacology & toxicology

A number of clinical studies are reported in the literature and have shown that HPBCD is well-tolerated and safe in the majority of patients receiving HPBCD at daily oral doses of the 4-8 g for at least two weeks (Irie, T and Uekama, K. (1977) J. Pharm. Sci 86, 147-162.). Higher daily doses of 16 g to 24 g used for 14 days in volunteers resulted in increased incidence of soft stools and diarrhea. Therefore based on these clinical data, HPBCD was considered to be non-toxic if an oral daily dose is less than 16 g. In an intravenous dose study, single doses up to 3 g were found to have no measurable effect on kidney function, and was well tolerated by all volunteers (Seiller et al. (1990) in: Duchene, D. (Ed). Minutes of the 5<sup>th</sup> International Symposium on cyclodextrins, Editions de Sante. Paris, pp 518-540). Following one week intravenous study at a dose level up to 1 g, no adverse effects were reported (Janssen Technical Bulletin, (1992). Encapsin HPB hydroxypropyl-beta-cyclodextrin. A real solution for real drug delivery problems. Janssen Biotech. N.V. pp 1-7).

The pharmacokinetics of HPBCD has been studied in healthy volunteers after single intravenous and oral dosing. Following intravenous doses at 0.5, 1.0, 1.5, 2.0, 2.5 or 3 g, plasma levels of the unchanged HPBCD declined rapidly and showed a bi-phasic decline. There were no differences between males and females and dose proportionality was demonstrated. Pharmacokinetic parameters such as half-life clearance and the V<sub>dss</sub> were shown to be independent of dose and urine levels suggesting that elimination was almost totally via the kidneys and there was no sign of tubular reabsorption. Following oral administration, HPBCD could not be detected in either the plasma after 1 hour or urine, indicating that there was no absorption from the GI tract and that oral bioavailability in humans was low (Szathmary, S.C et al. (1990) in: Duchene, D. (Ed). Minutes of the 5<sup>th</sup> International Symposium on cyclodextrins, Editions de Sante. Paris, pp 535-540).

2) Pediatric therapeutic use



Carpenter et.al.<sup>5</sup> reported treating a three year old hypervitaminosis A patient with infusions of HPBCD in an effort to solubilize retinoids and enhance their urinary excretion. The patient received a continuous intravenous infusion at 470 mg/kg/24hrs for a total of 30 g over 4 day period in the form of 5% aqueous solution in water. Other than generalized irritability and leg pain associated with vitamin A toxicity, no adverse events were reported, although cholesterol levels decreased by 20-30% during the cyclodextrin infusions.

HPBCD has been used in children as young as seven months of age as a solubilizer for the parenteral administration of anti-fungal agents. Abdel-Rahman et. al. (Antimicrobial Agents and Chemotherapy, 2007, pp2668-2673, vol 51 #8) safely administered itraconazole 2.5 mg/kg in 0.1 –g/kg HPBCD as a 1 hr intravenous infusion. The authors measured HPBCD pharmacokinetics and observed that levels fell below quantifiable limits by 12 hrs. The volume of distribution approximated the extracellular fluid space. The total plasma clearance of HPBCD approximated estimates of the glomerular filtration rate. The population estimated value of clearance (CL) was 5.27 liters/hr for a 30 kg child.

#### B. Scientific Rationale for Use of Hydroxy-Propyl-Beta-Cyclodextrin in the Treatment of Niemann-Pick Disease Type C

CHORI is seeking orphan designation for Hydroxy-Propyl-Beta-Cyclodextrin for treatment of genetically-defined NPC patients. The scientific rationale for the use of HPBCD in the treatment of NPC is predicated upon recent findings in an animal model of the disease. Niemann-Pick Disease Type C is a rare autosomal recessive lysosomal storage disease and is related to mutations of the NPC1 protein responsible for cholesterol trafficking between the late endosomal and lysosomal compartments to the cytosol of all cells. As a result, clinical manifestations are organ dysfunction and ultimately neurological decline secondary to neurodegeneration. A homozygous mutant mouse (NPC<sup>-/-</sup>) has been identified that manifests many of the same defects seen in humans. This model has been used to elucidate the various functioning of the NPC1 gene and to screen for compounds with potential therapeutic potential.

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<sup>5</sup> Carpenter et. al. (J. Pediatrics, 1987, pp507-512)



Liu et.al.<sup>6</sup> demonstrated that a single 4000 mg/kg s.c. injection of HPBCD at seven days of age prolonged the life of NPC-/- mutant mice. The effect size in that study, however, was a modest 15%. More recently, these authors<sup>7</sup> expanded their findings to show a single injection of HPBCD reduces whole-body burden of cholesterol by >900 mg/kg. Dr. Steve Walkley's group at Albert Einstein College of Medicine report (Abstract, Lysosomal Disease Network Annual Meeting, February 18-20, 2009) that NPC-/- mice treated daily from day seven with 4000 mg/kg HPBCD demonstrated a three week delay in onset of clinical symptoms and survived more than double that of untreated NPC-/- mice.

Camargo et.al.<sup>8</sup> found a similar small effect of HPBCD. They concluded the slight effects of the HPBCDs on neurological symptoms may be partially due to their apparent non-permeation of the blood-brain barrier (BBB). By contrast, recent unpublished work (C. Vite, personal communication, publication pending) indicates that 120 mg HPBCD given as intrathecal injections every two weeks delayed the onset of neurological symptoms in a feline model of NPC.

Recently, Davidson et. al.<sup>9</sup> reported that administration of HPBCD to NPC1 -/- mice beginning at either P7 or P21 and continuing every other day delayed clinical onset, reduced intra-neuronal cholesterol and ganglioside storage as well as free sphingosine accumulation, reduced markers of neurodegeneration, and led to longer survival than any previous treatment. Similar effects were seen in NPC2 -/- mice. Further, the authors demonstrated that the effects appear specific to NPC as cyclodextrin failed to alter cholesterol, ganglioside accumulation in animal models of MPSIIIA or GM1 gangliosidosis.

Mechanistically, it appears that cyclodextrin is capable of reversing the deficit in NPC1 and NPC2 cells resulting in the reversal of LDL-derived cholesterol accumulation in lysosomes that otherwise fail to

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<sup>6</sup> Liu et.al. (J. Lipid Res, 2008, vol 49, pp663-669)

<sup>7</sup> Liu et.al. (PNAS \_ February 17, 2009 \_ vol. 106 \_ no. 7 \_ 2377–2382)

<sup>8</sup> Camargo et.al. (Life Sci. 2001, 70(2):131-42)

<sup>9</sup> Davidson et al (PlosOne.org, vol 4 e6951)

deliver LDL cholesterol to the endoplasmic reticulum for esterification.<sup>10</sup>

To date, HPBCD appears to produce the greatest effect on survival and delay in the onset of neurological symptoms in animal models of NPC1 and NPC2. Based upon these profound findings and the relative safety of HPBCD, CHORI believes human clinical trials in NPC patients are warranted.

#### C. Use of Hydroxy-Propyl-Beta-Cyclodextrin in the Treatment of Niemann-Pick Disease Type C

Based upon the recent findings in animal models of NPC several children world-wide have been receiving i.v. infusions of Trappsol brand of HPBCD.

In January, 2009, CHORI filed an application for a Single Patient Investigational New Drug Exemption with the Division of Gastrointestinal Drug Products, requesting compassionate use of HPBCD in two children with NPC disease. These studies are on-going. A copy of the pertinent information is provided in Appendix 5.

Addi and Cassi Hempel (DOB 1/23/2004), identical twin Caucasian females, received a definitive diagnosis of NPC in October, 2007. The girls have been receiving miglustat (Zavesca®) 100 mg BID for almost one year. In addition, the children are receiving nutritional supplements including curcumin.

Both Hempel children have been participating in a study at the National Institutes of Health designed to track the progression of NPC disease. The children were last examined at the NIH in July of 2008. Both children continue to exhibit hepatosplenomegaly and clinical deterioration consistent with disease progression as manifested by severe neurological symptoms including cerebellar ataxia, dysarthria, dysphagia, vertical supranuclear palsy and cataplexy as well as progressive dementia.

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<sup>10</sup> Mosleh et al, (PNAS 106: 19316-19321, 2009)

On April 13, 2009, under the Single Patient INDs (Caroline Hastings, M.D. from CHORI acting as PI) the children began receiving intravenous infusions of Hydroxyl-Propyl-Beta-cyclodextrin (HPBCD) starting at a dose of 80 mg/kg/day. The dose of HPBCD had been escalated on a monthly basis to the initial target levels of 400 mg/kg/day administered as a weekly eight hour infusion in a hospital setting. Because the treatment has failed to result in significant observable clinical improvement or in adverse events, increased dosing frequency (twice/week) and rate of dose titration (100 g/kg/infusion) was increased beginning July 16, 2009.

The Hempel girls tolerated this therapy very well, without the anticipated renal or hepatic toxicities. A protocol amendment submitted on July 31, 2009, allowed for continued dose escalation by 100 mg/kg/dose, still given as 8-hour infusions twice a week. Toxicity monitoring continued as previous and again, no toxicities were found. On several occasions, the urine protein/creatinine ratio exceeded the normal level of 0.2 and the dose was decreased by 200 mg/kg and re-assessed. Each time, the levels returned to normal. It is thought that this test is not very predictive of underlying renal pathology as it is acutely affected by such factors as later morning collection and also diaper contamination.

In addition to monitoring for laboratory toxicities, the Hempel girls have been monitored for neurologic changes including ataxia, generalized tone, speech, and further development. Very importantly, the Hempel girls have not shown evidence of continued neurologic regression since initiation of the infusions. There have been noted improvements in general alertness, strength, and gait. These improvements were seen consistently by a dose of 1000 mg/kg and have been sustained. There have been no appreciable continued changes since dose escalation above 2500 mg/kg.

As of mid-October, 2009, it was decided to hold the dosing at 5000 mg/kg/ week, which is divided into Monday-Thursday infusions of 2500 mg/kg/dose. This was prompted by recent animal data in which a new concern of pulmonary interstitial disease was seen, though it remains uncertain if this is drug or dosing related, or possibly as a result of the natural history of the disease in an animal now living longer than expected. The infusions are stable while new baseline pulmonary studies are being obtained and a system of monitoring put in place.

As a part of the treatment plan blood, urine and fecal samples have been collected to examine the potential effects of HPBCD on various biomarkers. In fact, as a result of a pilot study of the use of HPBCD in treating NPC children, investigators observed changes toward normalization of disease-specific oxysterols and changes in the size of lysosomes. While these preliminary findings need to be confirmed and expanded, the observations suggest that HPBCD may provide effective therapy for NPC.

## **V. Previously Approved Drugs for Use in the Treatment of Niemann Pick Disease Type C**

*Where the sponsor of a drug that is otherwise the same drug as an already approved orphan drug seeks orphan drug designation for the subsequent drug for the same rare disease or condition, a sponsor's request for an orphan drug designation must contain an explanation of why the proposed variation may be clinically superior to the first drug. 21 C.F.R. § 316.20(b)(5).*

CHORI is not seeking orphan designation for a drug for which FDA has already granted orphan drug designation for Niemann-Pick Disease Type C. This provision is, therefore, inapplicable for purposes of the present Petition for Orphan Designation.

## **VI. Treatment of a Subset of Patients**

*Where a drug is under development for only a subset of persons with a particular disease or condition, a sponsor's request for an orphan drug designation must contain a demonstration that the subset is medically plausible. 21 C.F.R. § 316.20(b)(6).*

This Orphan Drug Petition seeks to obtain an orphan designation for Hydroxy-Propyl-Beta-Cyclodextrin for treatment of all patients with NPC.

## **VII. Hydroxy-Propyl-Beta-Cyclodextrin Regulatory Status and Marketing History**

*A sponsor's request for an orphan drug designation must contain a summary of the regulatory status and marketing history of the drug in*

*the United States and in foreign countries, e.g., IND and marketing application status and dispositions, what uses are under investigation and in what countries; for what indication is the drug approved in foreign countries; what adverse regulatory actions have been taken against the drug in any country. 21 C.F.R. § 316.20(b)(7).*

#### A. Regulatory Status

CHORI is unaware of any regulatory issues concerning HPBCD in general and Trappsol™ specifically.

- a) HPBCD has been approved in the United States for use as a pharmaceutical excipient in a number of different formulations. An excellent review of cyclodextrins in drug delivery can be found at:

<http://www.hi.is/~thorstlo/general.pdf> <sup>11</sup>

Examples of marketed products containing **2-Hydroxypropyl-β-cyclodextrin**

Cisapride	Rectal	Propulsid Europe
Hydrocortisone	Buccal	Dexocort Europe
Indomethacin	Eye drops	Indocid Europe
Itraconazole	Oral, IV	Sporanox Europe, USA
Mitomycin	IV	Mitozytrex USA

A summary of the previous human experience with HPBCD and potential risks to patients are summarized above, and animal toxicology summarized in Appendix 3. The safety and effectiveness in children under 12 years of age have not been demonstrated.

#### b. Marketing History

HPBCD has been marketed worldwide and is marketed in the United States as a pharmaceutical excipient.

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<sup>11</sup> <http://www.hi.is/~thorstlo/general.pdf>

To the best of CHORI's knowledge, investigational dosage forms have not been marketed in the United States or in any foreign market. CHORI is not aware that any patient received the investigational dosage forms in the United States prior to initiation of its clinical studies under IND Nos. 104,114 Addison Hempel and 104,116 Cassidy Hempel.

CHORI does not have any knowledge that any HPBCD product has been withdrawn from the United States or from any foreign market.

### **VIII. Documentation that Niemann-Pick Disease Type C Affects Fewer than 200,000 People in the United States**

*A sponsor's request for an orphan drug designation must contain documentation, with appended authoritative references, to demonstrate that the disease or condition for which the drug is intended affects fewer than 200,000 people in the United States or, if the drug is a vaccine, diagnostic drug, or preventive drug, the persons to whom the drug will be administered in the United States are fewer than 200,000 per year as specified in 21 C.F.R. § 316.21(b). 21 C.F.R. § 316.20(b)(8)(I).*

By submitting this Orphan Drug Petition, CHORI is seeking orphan designation for use of Hydroxy-Propyl-Beta-Cyclodextrin for the treatment of NPC, for the reasons enumerated. CHORI submits that the prevalence of genetically diagnosable NPC does not exceed 200,000 individuals in the United States. Thus, there are fewer than 200,000 NPC patients in the United States with NPC as of the date of this application.

On November 12, 2008 Orphan Product Designation was granted for miglustat (Zavesca®) for Treatment of the neurological manifestations of Niemann-Pick disease, type C.<sup>12</sup> This fact is further supporting information for NPC as an orphan designation.

CHORI believes it is appropriate to consider a prevalence range of NPC as 1/150,000 in the United States, as reported in most papers relying on

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<sup>12</sup> [http://www.accessdata.fda.gov/scripts/opdlisting/oopd/OOPD\\_Results\\_2.cfm?Index\\_Number=270708](http://www.accessdata.fda.gov/scripts/opdlisting/oopd/OOPD_Results_2.cfm?Index_Number=270708)

molecular diagnostic techniques<sup>13</sup>. The population of the United States on November 1, 2009 was approximately 300 million.<sup>14</sup> Using the prevalence data specified above for NPC as 1/150,000 results in an estimate of fewer than 2,000 patients in the United States with NPC; these estimated figures fall within the limit of 200,000 mandated in FDA's regulations. See 21 C.F.R. § 316.21(b).

The number of individuals for whom Hydroxy-Propyl-Beta-Cyclodextrin is indicated for treatment of the neurological manifestations of NPC is, therefore, within the limit set by FDA's orphan drug regulations. See 21 C.F.R. § 316.21(b). Therefore, CHORI need not submit documentation demonstrating that there is no reasonable expectation of recovering the costs of research and development of HPBCD for the indication described in this Petition. The prevalence of NPC, and the prevalence of the subpopulation expected to benefit from treatment with HPBCD is under 200,000 individuals.

## **IX. Statement Identifying the Real Party in Interest**

*A sponsor's request for an orphan drug designation must contain a statement as to whether the sponsor submitting the request is the real party in interest of the development and the intended or actual production and sales of the product.*

CHORI is the real party in interest in the development of Hydroxy-Propyl-Beta-Cyclodextrin for use in the treatment of patients with NPC. However, due to CHORI's limited financial resources, it is anticipated that future commercial development of HPBCD for the treatment of NPC may be transferred to an as yet unidentified biopharmaceutical company possessing greater drug development and marketing expertise.

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<sup>13</sup> Meikle et al JAMA 1999; 281(3):249-254. Prevalence of Lysosomal storage disorders.

<sup>14</sup> <http://www.census.gov/cgi-bin/popclock> . According to the U.S. Bureau of the Census, the resident population of the United States, projected to November 1, 2009, was 307 million.

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## APPENDIX 1

### NPC and HPBCD Research Article Summaries

1. <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=npc> Patterson.
2. Patterson et al. J. Child Neurol. Oct 12, 2009
3. Millat et al Am. J. Hum. Genet, 2001 a,b
4. Zervas et al Curr. Biol. 2001
5. Carpenter et. Al. (J. Pediatrics, 1987, pp507-512)
6. Liu et.al. (J. Lipid Res, 2008,vol 49, pp663-669)
7. Liu et.al. (PNAS \_ February 17, 2009 \_ vol. 106 \_ no. 7 \_ 2377–2382)
8. Camargo et.al. (Life Sci. 2001, 70(2):131-42)
9. Davidson et al (PlosOne.org, vol 4 e6951)
10. Mosleh et al,  
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## Niemann-Pick Disease Type C

[Juvenile Niemann-Pick Disease. Includes: Niemann-Pick Disease Type C1, Niemann-Pick Disease Type C2]

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### Summary

**Disease characteristics.** Niemann-Pick Disease Type C (NPC) is a lipid storage disease that can present in infants, children, or adults. Neonates can present with ascites and severe liver disease from infiltration of the liver and/or respiratory failure from infiltration of the lungs. Other infants, without liver or pulmonary disease, have hypotonia and developmental delay. The classic presentation occurs in mid-to-late childhood with the insidious onset of ataxia, vertical supranuclear gaze palsy (VSGP), and dementia. Dystonia and seizures are common. Dysarthria and dysphagia eventually become disabling, making oral feeding impossible; death usually occurs in the late second or third decade from aspiration pneumonia. Adults are more likely to present with dementia or psychiatric symptoms.

**Diagnosis/testing.** The diagnosis of NPC is confirmed by biochemical testing that demonstrates impaired cholesterol esterification and positive filipin staining in cultured fibroblasts. Biochemical testing for [carrier](#) status is unreliable. Most individuals with NPC have NPC1, caused by [mutations](#) in the *NPC1* [gene](#); fewer than 20 individuals have been diagnosed with NPC2, caused by [mutations](#) in the *NPC2* [gene](#). [Molecular genetic testing](#) of the *NPC1* and *NPC2* [genes](#) detects [disease-causing mutations](#) in approximately 94% of individuals with NPC. Such testing is available clinically.

**Management.** *Treatment of manifestations:* symptomatic therapy for seizures, dystonia, and cataplexy; a nocturnal sedative to help disordered

sleep; physical therapy to maintain mobility as long as possible. *Prevention of secondary complications:* chest physical therapy with aggressive bronchodilation and antibiotic therapy of intercurrent infection; regular bowel program for mobility-impaired individuals to prevent severe constipation and resulting increased seizure frequency and/or increased spasticity. *Surveillance:* Swallowing is monitored to allow placement of a gastrostomy tube when aspiration or nutritional compromise is imminent. *Agents/circumstances to avoid:* drugs that cause excessive salivation or that may exacerbate seizures by interacting with antiepileptic drugs; alcohol and other drugs that may exacerbate ataxia.

**Genetic counseling.** NPC is inherited in an autosomal recessive manner. Each sib of an affected individual has a 25% chance of being affected, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier. The phenotype (i.e., age of onset and severity of symptoms) usually runs true in families. Carrier testing for at-risk relatives and prenatal testing for pregnancies at increased risk are possible when the two disease-causing mutations have been identified in the family.

## **Diagnosis**

### **Clinical Diagnosis**

The diagnosis of Niemann-Pick Disease Type C (NPC) should be considered in individuals presenting with the following [[Vanier 1997](#)]:

- Fetal ascites or neonatal liver disease, particularly when the latter is accompanied by prolonged jaundice and pulmonary infiltrates
- Infantile hypotonia without evidence of progression for months to years, followed by features outlined in [Brady et al \[1989\]](#) (see VSPG)
- Vertical supranuclear gaze palsy (VSPG), followed by progressive ataxia, dysarthria, dystonia, and, in some cases, seizures and gelastic cataplexy, beginning in middle childhood, and progressing slowly over many years. Rarely, such presentations may begin later in childhood or in adulthood.
- Psychiatric presentations, mimicking depression or schizophrenia, with few or subtle neurologic signs, beginning in adolescence or adulthood
- Enlargement of the liver or spleen, particularly in early childhood

## **Testing**

**Biochemical.** For laboratories offering biochemical testing, see [Testing](#).

Definitive diagnosis of NPC requires demonstration of abnormal intracellular cholesterol homeostasis in cultured fibroblasts [[Pentchev et al 1985](#)]. These cells show reduced ability to esterify cholesterol after loading with exogenously derived LDL-cholesterol. Filipin staining demonstrates an intense punctate pattern of fluorescence concentrated around the nucleus, consistent with the accumulation of unesterified cholesterol:

- **Classic.** Most individuals have zero or very low esterification levels with a classic staining pattern.
- **Variant.** About 15% of individuals have intermediate or 'variant' levels of cholesterol esterification and a less distinctive staining pattern. More precise characterization of the biochemical defect in this group can be achieved by the use of BODIPY-lactosylceramide to identify lipid trafficking abnormalities [[Sun et al 2001](#)]. Such testing is currently available only on a research basis.

**Histology.** Other tests, including tissue biopsies and tissue lipid analysis, which were essential for diagnosis before recognition of the biochemical defect in NPC, are now rarely needed. These tests include examination of bone marrow, spleen, and liver, which contain foamy cells (lipid-laden macrophages); sea-blue histiocytes may be seen in the marrow in advanced cases. Electron microscopy of skin, rectal neurons, liver, or brain may show polymorphous cytoplasmic bodies [[Boustany et al 1990](#)].

## Molecular Genetic Testing

*GeneReviews designates a molecular genetic test as clinically available only if the test is listed in the [GeneTests Laboratory Directory](#) by either a US CLIA-licensed laboratory or a non-US clinical laboratory. GeneTests does not verify laboratory-submitted information or warrant any aspect of a laboratory's licensure or performance. Clinicians must communicate directly with the laboratories to verify information.*—ED.

**Genes.** Two [genes](#) are associated with Niemann-Pick Disease Type C (NPC):

- ***NPC1*.** From complementation studies and [linkage analysis](#), it is concluded that the majority of individuals with NPC harbor [mutations](#) in the *NPC1* [gene](#).

- ***NPC2***. It is assumed that the remaining individuals with the NPC [phenotype](#) have [mutations](#) in *NPC2*. *NPC2* [mutations](#) have been detected in 4% of individuals with NPC [[Park et al 2003](#)].

**Other loci.** No direct evidence exists for other loci; however, in some individuals with the typical clinical and biochemical [phenotype](#), [mutations](#) have not been found in *NPC1* or *NPC2*.

## Clinical testing

- **[Sequence analysis](#)**. Detection rates using [sequence analysis](#) may be comparable to those found using [mutation scanning](#), which has identified *NPC1* [mutations](#) in 90% [[Park et al 2003](#)] and *NPC2* [mutations](#) in 4% of individuals with NPC:
  - Most individuals with *NPC1* are [compound heterozygotes](#) with [mutations](#) unique to their family; to date, [mutations](#) in one or both *NPC1* [alleles](#) cannot be identified in a substantial number of cases [[Greer et al 1999](#), [Yamamoto et al 1999](#), [Park et al 2003](#)].
  - Of note, individuals with *NPC1* from Nova Scotia (previously said to have Niemann-Pick type D) almost uniformly have the [p.Gly992Trp mutation](#) [[Greer et al 1998](#)].
  - The majority of identified [mutations](#) are missense alterations, raising the question of whether some of these could be benign [polymorphisms](#) or variants rather than pathogenic [mutations](#).
- **[Deletion/duplication analysis](#)**. No large [insertions](#) or [deletions](#) have been reported in *NPC2*. Based on the high [sensitivity](#) of the *NPC2* sequencing test, a [screening](#) test for large [deletions/duplications](#) is expected to have a very low yield.

[Table 1](#) summarizes [molecular genetic testing](#) for this disorder.

Table 1. [Molecular Genetic Testing](#) Used in Niemann-Pick Disease Type C

<a href="#">Gene Symbol</a>	Proportion of NPC Attributed to <a href="#">Mutations</a> in This	Test Method	<a href="#">Mutations</a> Detected	<a href="#">Mutation</a> Detection Frequency by <a href="#">Gene</a> and Test Method	Test Availability
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<u>Gene</u> <sup>1</sup>					
<i>NPC1</i>	90%	<a href="#">Sequence analysis</a>	Sequence variants	80%-90%	
		<a href="#">Duplication/deletion testing</a> <sup>2</sup>	Partial and whole <a href="#">gene deletions</a>	Unknown <sup>3</sup>	Clinical <a href="#">Testing</a>
<i>NPC2</i>	4%	<a href="#">Sequence analysis</a>	Sequence variants	Close to 100%	
		<a href="#">Duplication/deletion testing</a> <sup>2</sup>	Partial and whole <a href="#">gene deletions</a>	Unknown <sup>4</sup>	Clinical <a href="#">Testing</a>

1. Percent of individuals with NPC who have at least one identifiable [mutation](#) [Greer et al 1999, Yamamoto et al 1999, Park et al 2003] using a [mutation scanning](#) testing method.
2. Testing that detects [deletions/duplications](#) not readily detectable by [sequence analysis](#) of genomic [DNA](#); a variety of methods including [quantitative PCR](#), real-time [PCR](#), multiplex ligation dependent [probe](#) amplification (MLPA), or array CGH may be used.
3. Few have been reported; the frequency of such [mutations](#) may be rare.
4. No large [insertions](#) or [deletions](#) have been reported in NPC2. Based on the high [sensitivity](#) of the NPC2 sequencing test, a [screening](#) test for large [deletions/duplications](#) may have a very low yield.

**Interpretation of test results.** For issues to consider in interpretation of [sequence analysis](#) results, click [here](#).

## Testing Strategy

### Establishing the diagnosis in a [proband](#)

- Biochemical testing demonstrating abnormal intracellular cholesterol homeostasis in cultured fibroblasts is the mainstay of diagnosis and may be supported by ultrastructural changes on skin or rectal biopsy.

- [Molecular genetic testing](#) is used primarily to confirm the diagnosis in individuals with variant biochemical findings.

**[Carrier testing](#) for at-risk relatives** requires prior identification of the [disease-causing mutations](#) in the family.

Note: [Carriers](#) are [heterozygotes](#) for this [autosomal recessive](#) disorder and are not at risk of developing the disorder.

**[Prenatal diagnosis](#) and preimplantation genetic diagnosis** for at-risk pregnancies require prior identification of the [disease-causing mutations](#) in the family.

### **Genetically Related (Allelic) Disorders**

No other [phenotypes](#) are known to be associated with [mutations](#) in the *NPC1* and *NPC2* [genes](#).

### **Clinical Description**

#### **Natural History**

Niemann-Pick Disease Type C (NPC) may present at any age.

**Neonatal and infantile presentations.** The presentation of NPC in early life is nonspecific and may go unrecognized by inexperienced clinicians. On occasion, ultrasound examination in late pregnancy has detected fetal ascites; infants thus identified typically have severe neonatal liver disease with jaundice and persistent ascites.

Infiltration of the lungs with foam cells may accompany neonatal liver disease or occur as a primary presenting feature (pulmonary failure secondary to impaired diffusion).

Many infants succumb at this stage. Of those who survive, some are hypotonic and delayed in psychomotor development, whereas others may have complete resolution of symptoms, only to present with neurologic disease many years later. Liver and spleen are enlarged in children with symptomatic hepatic disease; however, children who survive often 'grow into their organs,' so that organomegaly may not be detectable later in

childhood. Indeed, many individuals with NPC never have organomegaly. The absence of organomegaly never eliminates the diagnosis of NPC.

Another subgroup of children has minimal or absent hepatic or pulmonary dysfunction and presents primarily with hypotonia and delayed development. Children in this group usually do not have vertical supranuclear gaze palsy (VSGP) at the onset but acquire this sign after a variable period, when other evidence of progressive encephalopathy supervenes.

**Childhood presentations.** The classic presentation of NPC is in middle-to-late childhood, with clumsiness and gait disturbance that eventually become frank ataxia. Many observant parents are aware of impaired vertical gaze, which is an early manifestation. VSGP first manifests as increased latency in initiation of vertical saccades, after which saccadic velocity gradually slows and is eventually lost. In late stages of the illness, horizontal saccades are also impaired. The physical manifestations are accompanied by insidiously progressive cognitive impairment, often mistaken at first for simple learning disability. Some children are thought to have primary behavioral disturbances, reflecting unrecognized dyspraxia in some instances. As the disease progresses, it becomes clear that the child is mentally deteriorating.

In addition to the manifestations outlined above, many children develop dystonia, typically beginning as action dystonia in one limb and gradually spreading to involve all of the limbs and axial muscles. Speech gradually deteriorates, with a mixed dysarthria and dysphonia. Dysphagia progresses in parallel with the dysarthria, and oral feeding eventually becomes impossible.

Approximately one-third of individuals with NPC have partial and/or generalized seizures. Epilepsy may be refractory to medical therapy in some cases. Seizures usually improve if the child's survival is prolonged, this improvement presumably reflecting continued neuronal loss. About 20% of children with NPC have gelastic cataplexy, a sudden loss of muscle tone evoked by a strong emotional (humorous) stimulus. This can be disabling in those children who experience daily multiple attacks during which injuries may occur.

Mild demyelinating peripheral neuropathy has been described in a child with otherwise typical late-infantile NPC [[Zafeiriou et al 2003](#)]. This finding is



likely a rare manifestation of NPC because prospective nerve conduction studies in a cohort of 41 [affected](#) individuals participating in a clinical trial of miglustat have identified only one case to date [Patterson 2006, personal communication].

Polysomnographic and biochemical studies have demonstrated disturbed sleep and variable reduction in cerebrospinal fluid hypocretin concentration in individuals with NPC, suggesting that the disease could have a specific impact on hypocretin-secreting cells of the hypothalamus [[Kanbayashi et al 2003](#), [Vankova et al 2003](#)].

Death from aspiration pneumonia usually occurs in the late second or third decade.

**Adolescent and adult presentations.** Adolescents or adults may present with neurologic disease as described in the preceding section, albeit with a much slower rate of progression. The author has seen one individual who survived into the seventh decade, having first developed symptoms 25 years earlier. Older individuals may also present with apparent psychiatric illness [[Imrie et al 2002](#), [Josephs et al 2003](#)], sometimes appearing to have major depression or schizophrenia. The psychiatric manifestations may overshadow neurologic signs, although the latter can usually be detected with careful examination. An adult presenting with bipolar disorder has been described [[Sullivan et al 2005](#)].

A German report describes two individuals with adult-onset dementia associated with frontal lobe atrophy and no visceral manifestations, as is common in adult-onset disease [[Klunemann et al 2002](#)].

**Imaging.** MRI of the brain is usually normal until the late stages of the illness. At that time, marked atrophy of the superior/anterior cerebellar vermis, thinning of the corpus callosum, and mild cerebral atrophy may be seen. Increased signal in the peritrial white matter, reflecting secondary demyelination, may also occur. In one adult, areas of confluent white matter signal hyperintensity mimicked multiple sclerosis [[Grau et al 1997](#)].

Limited studies of magnetic resonance spectroscopy (MRS) suggest that MRS may be a more sensitive imaging technique in NPC than standard MRI [[Tedeschi et al 1998](#)].



**Heterozygotes.** A recent report described an NPC1 [heterozygote](#) with tremor that the authors attributed to the mutant [allele](#) [Josephs et al 2004]. This observation notwithstanding, the question of manifesting [heterozygotes](#) must remain moot pending systematic prospective studies.

## Genotype-Phenotype Correlations

In the approximately 200 [mutations](#) described in *NPC1* [Scott & Ioannou 2004, Fernandez-Valero et al 2005], [genotype-phenotype correlation](#) is limited because most [affected](#) individuals are [compound heterozygotes](#); and correlation of the trafficking defects demonstrable in culture and the clinical [phenotype](#) is poor. Nonetheless, some correlations have been possible for homozygous [mutations](#) and the more common [mutations](#) in heterozygous state:

### *NPC1*

- One international study documented [phenotypes](#) associated with a [mutation](#) leading to a [p.Ile1061Thr](#) change in the Hispanic population in the upper Rio Grande Valley in the southwestern US, and in the UK and France. No individuals with this [mutation](#) had the severe infantile form of NPC [Millat et al 1999].
- More recently, the same group found that premature-termination-codon [mutations](#), [mutations](#) involving the sterol-sensing [domain](#), and [p.Ala1054Thr](#) in the cysteine-rich luminal loop of *NPC1* are associated with early-onset disease and classic biochemical changes [Millat et al 2001b].
- All mutant [alleles](#) that correlate with the biochemical 'variant' [phenotype](#) are clustered in the cysteine-rich luminal loop [Millat et al 2001b].
- A study of 40 unrelated individuals of Spanish descent suggested that those homozygous for the [p.Gln775Pro mutation](#) showed a severe infantile neurologic form and those homozygous for the [p.Cys177Tyr mutation](#), a late-infantile clinical [phenotype](#) [Fernandez-Valero et al 2005].

### *NPC2*

Of the five [mutations](#) identified by Millat et al [2001b], all but [c.190+5G>A](#) were associated with a severe [phenotype](#), characterized by pulmonary infiltrates, respiratory failure, and death by age four years:

- The two individuals with splice site [mutations](#) had juvenile-onset disease and prolonged survival.
- Adult-onset disease with frontal lobe atrophy has been described in association with a [p.Val39Met mutation](#) in *NPC2* [[Klunemann et al 2002](#)].
- Neonatal or infantile onset and death in early childhood were reported in children homozygous for [p.Gln45X](#), [p.Cys47X](#), and [p.Cys99Arg](#), whereas prolonged survival into middle adult life has been seen in those homozygous for p.Val39Met and [p.Ser67Pro](#) [[Chikh et al 2005](#)].

## Nomenclature

The older literature on NPC is bedeviled by the large number of terms used to describe individuals now known to have the disease. These include juvenile dystonic idiocy, juvenile dystonic lipidosis, juvenile NPC, neurovisceral lipidosis with vertical supranuclear gaze palsy, Neville-lake disease, sea-blue histiocytosis, lactosylceramidosis, and DAF (downgaze paralysis, ataxia, foam cells) syndrome.

The term Niemann-Pick disease type D describes a genetic isolate from Nova Scotia that is biochemically and clinically indistinguishable from NPC and that also results from [mutation](#) of the *NPC1* [gene](#).

The terms NPC1 and NPC2 are now preferred because they accurately describe the mutated [genes](#) responsible for the [phenotype](#).

## Prevalence

The prevalence of NPC has been estimated at 1:150,000 in Western Europe. The prevalence of NPC in early life is probably underestimated, owing to its nonspecific presentations.

Acadians in Nova Scotia, individuals of Hispanic descent in parts of Colorado and New Mexico, and a Bedouin group in Israel represent genetic isolates with a [founder effect](#).

## Differential Diagnosis

*For current information on availability of genetic testing for disorders included in this section, see [GeneTests Laboratory Directory](#). —ED.*

**Neonatal and infantile presentations** include biliary atresia, [congenital infections](#), [alpha-1-antitrypsin deficiency](#), [tyrosinemia](#), malignancies (leukemia, lymphoma, histiocytosis), other storage diseases (e.g., [Gaucher disease](#), [Niemann-Pick disease type A](#), [Niemann-Pick disease type B](#)), and infections (e.g., TORCH). A study from Colorado found that 27% of infants initially diagnosed with idiopathic neonatal cholestasis and 8% of all infants with cholestasis had NPC [[Yerushalmi et al 2002](#)]. Although this cohort may have been enriched by a local Hispanic genetic isolate, the importance of Niemann-Pick Disease Type C (NPC) as a cause of jaundice in infants is appropriately emphasized.

**Childhood presentations** include pineal region or midbrain tumors causing dorsal midbrain syndrome, hydrocephalus, [GM2 gangliosidosis](#), [mitochondrial diseases](#), [maple syrup urine disease](#), attention-deficit disorder, learning disabilities, absence seizures, other dementing illnesses, [idiopathic torsion dystonia](#), [dopa-responsive dystonia](#), [Wilson disease](#), amino acidurias and organic acidopathies (e.g., glutaric aciduria type 1) (see [The Organic Acidemias: An Overview](#)), pseudodementia (depressive disorder), [neuronal ceroid-lipofuscinosis](#), subacute sclerosing panencephalitis (see [Mitochondrial DNA-Associated Leigh Syndrome and NARP](#)), HIV encephalopathy, sleep disorders, syncope, and periodic paralysis (see [Hyperkalemic Periodic Paralysis Type 1](#), [Hypokalemic Periodic Paralysis](#)).

**Adolescent and adult presentations** include [Alzheimer disease](#), Pick disease (an adult-onset disorder with dementia associated with characteristic neuronal inclusions called Pick bodies, not related to Niemann-Pick disease), [frontotemporal dementias](#), Steele-Richardson-Olzewski syndrome (also known as progressive supranuclear palsy), late-onset lysosomal storage diseases, syphilis, HIV dementia, and primary psychiatric illnesses.

## **Management**

### **Evaluations Following Initial Diagnosis**

To establish the extent of disease in an individual diagnosed with Niemann-Pick Disease Type C (NPC), the following evaluations are recommended:

- Assessment of ability to walk and transfer, manage secretions, and communicate (language, speech, and hearing)
- For individuals with hepatosplenomegaly, complete blood count and tests of hepatic function.

- MRI of the head; usually performed in the course of the workup and usually normal until the disease is advanced
- Consideration of EEG and sleep studies if the history suggests seizures or sleep disturbances

## **Treatment of Manifestations**

No definitive therapy for NPC exists.

Symptomatic therapy may be at least partially effective in the management of seizures, dystonia, and cataplexy.

If disordered sleep is identified, a nocturnal sedative may be indicated. In complex cases, formal evaluation by a sleep specialist should be considered.

Bronchoalveolar lavage has been described as effective in improving function in one child with pulmonary infiltrates [[Palmeri et al 2005](#)].

General supportive care, including respite for primary caregivers, is crucial to the maintenance of the family unit in the face of this devastating illness.

## **Prevention of Secondary Complications**

Chest physical therapy with aggressive bronchodilation and antibiotic therapy for intercurrent infection appears beneficial, although no systematic study has been performed.

Individuals whose mobility is compromised should have a regular bowel program to prevent severe constipation, which may present as increased seizure frequency or increased spasticity in some impaired individuals with NPC.

Physical therapy is indicated to maintain mobility as long as possible.

Swallowing must be monitored to allow consideration of gastrostomy tube placement when aspiration or nutritional compromise is imminent.

## **Surveillance**

General pediatric evaluations, with special attention to pulmonary function, swallowing, bowel habit, and mood (for occult depression) at six-month

intervals are appropriate for most juvenile and adult [affected](#) individuals. Sleep disturbances are common in NPC; the [affected](#) individual or caregiver should be questioned regarding sleep hygiene as a part of regular evaluation.

Annual psychometric testing may be helpful in arranging appropriate school or work placement.

Teenagers and adults with motor or sensory impairments who are driving should be monitored at six- to 12-month intervals to ensure that they do not present a risk to themselves or others.

### **Agents/Circumstances to Avoid**

Drugs that cause excessive salivation or that may exacerbate seizures directly by interacting with antiepileptic drugs should be avoided.

Alcohol as well as many drugs exacerbate ataxia and should be avoided.

### **Testing of Relatives at Risk**

See [Genetic Counseling](#) for issues related to testing of at-risk relatives for [genetic counseling](#) purposes.

### **Therapies Under Investigation**

Inhibition of glycosphingolipid synthesis by n-butyldeoxynojirimycin has been shown to delay onset and prolong survival in a murine model of NPC [[Zervas et al 2001](#)], and a therapeutic trial of the same agent is presently in progress in humans. Preliminary data show evidence of stabilization or benefit in some individuals [[Patterson et al 2007](#)].

Recent laboratory studies of cellular and murine models of NPC raise the possibility of small-molecule therapies to interdict pathways triggering apoptosis and related routes to cell death and dysfunction [[Patterson & Platt 2004](#)].

Preliminary studies of neurosteroid replacement therapy with allopregnenolone in NPC mice suggest similar improvements in survival to those seen with n-butyldeoxynojirimycin, provided that the steroid is administered early in postnatal life [[Mellon & Griffin 2002](#)]. Confirmatory studies are in progress [[Mellon et al 2008](#)].

Studies in tissue culture have demonstrated that direct or indirect over-expression of the GTPase Rab 9 reverses the NPC [phenotype](#) [[Choudhury et al 2002](#), [Walter et al 2003](#)]. Although not yet applicable in human trials, this finding suggests the existence of alternate pathways for mobilization of endosomal cargoes that are potential targets for small-molecule therapies.

[Screening](#) of a library of more than 44,000 compounds led to identification of a compound that corrects the NPC [phenotype](#) in cell culture [[Liscum et al 2002](#)]. It is not known if further development of this compound as a potential therapy is planned.

Search [ClinicalTrials.gov](#) for access to information on clinical studies for a wide range of diseases and conditions.

## **Other**

In the C57 murine model of NPC, all treatment modalities, including bone marrow transplantation, combined bone marrow and liver transplantation, and aggressive cholesterol-lowering therapy, have proven ineffective.

Although a trial of cholesterol-lowering agents showed that the amount of free cholesterol in the liver of individuals with NPC could be reduced by the administration of cholestyramine, lovastatin, and nicotinic acid [[Patterson et al 1993](#)], there is no evidence that this approach modifies the neurologic progression of NPC.

Liver transplantation in humans corrects hepatic dysfunction but does not ameliorate the neurologic disease.

**Genetics clinics**, staffed by genetics professionals, provide information for individuals and families regarding the natural history, treatment, [mode of inheritance](#), and genetic risks to other family members as well as information about available consumer-oriented resources. See the [GeneTests Clinic Directory](#).

See [Consumer Resources](#) for disease-specific and/or umbrella support organizations for this disorder. These organizations have been established for individuals and families to provide information, support, and contact with other affected individuals.

## **Genetic Counseling**

*[Genetic counseling](#) is the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions. The following section deals with genetic [risk assessment](#) and the use of [family history](#) and genetic testing to clarify genetic status for family members. This section is not meant to address all personal, cultural, or ethical issues that individuals may face or to substitute for consultation with a genetics professional. To find a genetics or [prenatal diagnosis](#) clinic, see the [GeneTests Clinic Directory](#).*

## **Mode of Inheritance**

Niemann-Pick disease type C (NPC) is inherited in an [autosomal recessive](#) manner.

## **Risk to Family Members**

### **Parents of a [proband](#)**

- Parents of children with NPC are [obligate heterozygotes](#).
- [Heterozygotes](#) are asymptomatic.

### **Sibs of a [proband](#)**

- At conception, each sib of an [affected](#) individual has a 25% chance of being [affected](#), a 50% chance of being an asymptomatic [carrier](#), and a 25% chance of being [unaffected](#) and not a [carrier](#). The [phenotype](#) usually runs true in families; that is, if the [proband](#) has early-onset disease, subsequent [affected](#) individuals will have a similar clinical course. In rare cases, a [proband](#) and subsequent offspring have had different clinical presentations.
- Sibs younger than the [proband](#) may have the disease but be asymptomatic. Assuming that the [phenotype](#) runs true in the family, a [proband](#)'s [unaffected](#) older sibs have a 2/3 risk of carrying one abnormal [allele](#) of an [NPC gene](#).

**Offspring of a [proband](#).** The offspring of an individual with NPC will inherit one abnormal [allele](#) of an [NPC gene](#) from the [affected](#) parent and are thus [obligate heterozygotes](#).



**Other family members of a [proband](#).** Each sib of a [proband](#)'s parents is at a 50% risk of being a [carrier](#).

## **Carrier Detection**

Biochemical testing is unreliable in defining the heterozygous state, owing to significant overlaps with findings seen in controls.

Molecular analysis of the *NPC1* or *NPC2* [gene](#) may be used for [carrier testing](#) if [mutations](#) have been identified in the *NPC1* or *NPC2* [gene](#) in the family.

## **Related Genetic Counseling Issues**

### **Family planning**

- The optimal time for determination of genetic risk, clarification of [carrier](#) status, and discussion of the availability of prenatal testing is before pregnancy.
- It is appropriate to offer [genetic counseling](#) (including discussion of potential risks to offspring and reproductive options) to young adults who are [affected](#), [carriers](#), or at-risk of being [carriers](#).

**[DNA banking](#).** [DNA banking](#) is the storage of [DNA](#) (typically extracted from white blood cells) for possible future use. Because it is likely that testing methodology and our understanding of [genes](#), [mutations](#), and diseases will improve in the future, consideration should be given to banking [DNA](#) of [affected](#) individuals. [DNA banking](#) is particularly relevant in situations in which the [sensitivity](#) of currently available [molecular genetic testing](#) is less than 100%. See [Testing](#) for a list of laboratories offering [DNA banking](#).

### **Prenatal Testing**

Prenatal testing is available for pregnancies at 25% risk for NPC using [molecular genetic testing](#) of [DNA](#) extracted from fetal cells obtained by amniocentesis usually performed at approximately 15-18 weeks' gestation or chorionic villus sampling (CVS) at approximately ten to 12 weeks' gestation. Both disease-causing [alleles](#) in a family must be identified before prenatal testing can be performed [[Vanier 2002](#)].



Note: Gestational age is expressed as menstrual weeks calculated either from the first day of the last normal menstrual period or by ultrasound measurements.

**Preimplantation genetic diagnosis (PGD).** Preimplantation genetic diagnosis may be available for families in which the [disease-causing mutations](#) have been identified. For laboratories offering PGD, see [Testing](#).

## Molecular Genetics

*Information in the Molecular Genetics and OMIM tables may differ from that elsewhere in the GeneReview: tables may contain more recent information.* —ED.

Table A. Niemann-Pick Disease Type C: Genes and Databases

Gene Symbol	Chromosomal Locus	Protein Name	HGMD
<a href="#">NPC1</a>	<a href="#">18q11-q12</a>	<a href="#">Niemann-Pick C1 protein</a>	<a href="#">NPC1</a>
<a href="#">NPC2</a>	<a href="#">14q24.3</a>	<a href="#">Epididymal secretory protein E1</a>	<a href="#">NPC2</a>

Data are compiled from the following standard references: gene symbol from [HGNC](#); chromosomal locus, locus name, critical region, complementation group from [OMIM](#); protein name from [UniProt](#). For a description of databases (Locus Specific, HGMD) linked to, click [here](#).

Table B. OMIM Entries for Niemann-Pick Disease Type C ([View All in OMIM](#))

[257220](#) NIEMANN-PICK DISEASE, TYPE C1; NPC1

[601015](#) NPC2 GENE; NPC2

[607623](#) NPC1 GENE; NPC1

[607625](#) NIEMANN-PICK DISEASE, TYPE C2

## Molecular Genetic Pathogenesis

The central defect in NPC is in intracellular trafficking of lipids, as opposed to the lysosomal hydrolase deficiency characteristic of the classic lysosomal storage diseases (LSDs). Notwithstanding, all LSDs are marked by the

accumulation of multiple lipid species in the lysosomes, and secondary trafficking impairment occurs in disorders with primary hydrolase deficiencies. In NPC, cholesterol accumulates in great excess in the lysosomes and may lead to a deficiency in membrane cholesterol. Given the critical role of cholesterol in maintaining membrane order, this downstream deficiency could conceivably play a role in membrane dysfunction, and possibly in the triggering of apoptosis [[Mukherjee & Maxfield 2004](#)].

Glycosphingolipid accumulation is characteristic of the neuropathology of NPC; animal studies have demonstrated that GM2 accumulation is associated with ectopic dendritogenesis and meganeurite formation, which — together with the formation of neurofibrillary tangles (cholesterol dysregulation) and neuroaxonal dystrophy — are likely anatomic substrates for neurologic dysfunction [[Walkley & Suzuki 2004](#)].

Trafficking studies suggest that NPC2 binds cholesterol in the luminal space of the late-endosome/lysosome and transports it to the delimiting membrane. NPC resides in the membrane of the late endosomes and is shuttled between that compartment and the plasma membrane and other internal sites. It remains to be determined how these two molecules interact, how they sense the presence and concentration of lipids, and why NPC1 accompanies its vesicular cargo to its destination [[Liscum & Sturley 2004](#)].

## ***NPC1***

**Normal allelic variants:** *NPC1* contains 25 [exons](#), varying in size from 74 to 788 bp, spread over 47 kb [[Morris et al 1999](#)]. More than 50 exonic [polymorphisms](#) have been described; the most prevalent are listed in [Table 2](#) [[Millat et al 2005](#)].

**Pathologic allelic variants:** Approximately 200 [mutations](#) have been described in *NPC1* [[Scott & Ioannou 2004](#), [Fernandez-Valero et al 2005](#)].

A study of 143 unrelated individuals with NPC identified 121 different [mutations](#) in 251 of 286 disease [alleles](#), an overall detection rate of 88% [[Park et al 2003](#)]. Cases negative for [mutations](#) showed a high proportion of equivocal results in complementation studies, raising the possibilities of (1) a third complementation group for NPC or (2) nonspecificity of NPC biochemical testing. The region between amino acids 1038 and 1253 (which includes the Patched 1 [domain](#)) and the region in amino acids identical to the NPC1 homolog NPC1L1 were hot spots for [mutations](#).

Most [affected](#) individuals are [compound heterozygotes](#) for [point mutations](#) producing missense (~70% of [mutations](#) overall) [[Millat et al 2005](#)] and [nonsense mutations](#); [deletions](#) and splice site [mutations](#) have also been reported.

A [mutation](#) leading to a p.Gly992Trp change has been identified in several individuals in the Acadian population of Nova Scotia [[Greer et al 1998](#)] and in Portugal; a p.Gly992Arg [mutation](#) has been described in France [[Fernandez-Valero et al 2005](#)] ([Table 2](#)).

A Spanish report found that individuals homozygous for the p.Gln775Pro [mutation](#) had a severe infantile neurologic illness, and those with the p.Cys177Tyr [mutation](#) had a late-infantile clinical [phenotype](#) [[Fernandez-Valero et al 2005](#)].

The p.Ile1061Thr [mutation](#) accounts for 15%-20% of mutated [alleles](#) in Western Europe and the US, followed by p.Pro1007Ala [[Millat et al 2005](#)].

Table 2. *NPC1* Allelic Variants Discussed in This *GeneReview*

<b><u>Class of Variant Allele</u></b>	<b><u>DNA Nucleotide Change (Alias <sup>1</sup>)</u></b>	<b><u>Protein Amino Acid Change</u></b>	<b><u>Reference Sequence</u></b>
Normal	c.709C>T	p.Pro237Ser	
	c.1926C>G	p.Ile642Met	
	c.2572A>G	p.Ile858Val	
	c.2793C>T	p.Asn93	
	c.3797G>A	p.Arg1266Gln	
	c.530G>A	p.Cys177Tyr	
	c.2324A>C	p.Gln775Pro	<a href="#">NM_000271.3NP_000262.1</a>
Pathologic	c.2974G>T	p.Gly992Trp	
	c.2974G>C	p.Gly992Arg	
	c.2974G>A	p.Gly992Arg	
	c.3019C>G	p.Pro1007Ala	
	c.3160G>A	p.Ala1054Thr	
	c.3182T>C	p.Ile1061Thr	

See [Quick Reference](#) for an explanation of nomenclature. *GeneReviews* follows the standard naming conventions of the Human [Genome](#) Variation Society (<http://www.hgvs.org>).

1. Variant designation that does not conform to current naming conventions

**Normal [gene product](#):** The *NPC1* [gene product](#) is an integral membrane protein with 13 transmembrane [domains](#), which appears to be localized to a late endosomal compartment. Its function is as yet imperfectly understood, but it clearly plays a central role in modulating intracellular sorting of cholesterol and glycosphingolipids [[Neufeld et al 1999](#)]. [Wojtanik and Liscum \[2003\]](#) have shown that in the cells of individuals with NPC1, LDL cholesterol traffics directly through endosomes to lysosomes, bypassing the plasma membrane, and is trapped there because of dysfunctional NPC1. NPC1 appears to serve this function, at least in part, by maintaining the small size of cholesterol-containing lipid droplets in the cell [[Wiegand et al 2003](#)]. [Strauss et al \[2002\]](#) have suggested that NPC1 may act cooperatively with NPC2 and MLN64 in an ordered sequence to effect intracellular sterol movement. Sterol storage in fibroblasts correlates with oxysterol levels; administration of oxysterols corrects the [phenotype](#) in cells with the p.Ile1061Thr [mutation](#), suggesting that NPC1 and NPC2 regulate intracellular sterol homeostasis via oxysterols [[Frolov et al 2003](#)]. [Domains 3-7](#) of the NPC1 protein have homology to the sterol-sensing [domains](#) of SCAP and HMG CoA reductase, and other [domains](#) are homologous to the *Drosophila* morphogen patched [[Carstea et al 1997](#)]. Studies in cultured fibroblasts have shown that specific [point mutations](#) in the sterol-sensing [domain](#) can induce either loss of function (p.Pro692Ser) or gain of function (p.Asp787Asn, p.Leu657Phe) in trafficking to the plasma membrane and ER [[Millard et al 2005](#)]. The overrepresentation of pathogenic [mutations](#) in these [domains](#) further emphasizes their key roles in the function of the protein (see [NPC1, Pathologic allelic variants](#)). NPC1 appears to mediate fatty acid transport in *E. coli*; but this is not the case in human NPC fibroblasts, where fatty acid trafficking is normal [[Passeggio & Liscum 2005](#)].

**Abnormal [gene product](#):** Deficiency of the *NPC1* [gene product](#) leads to a complex pattern of intracellular lipid storage, including excess unesterified cholesterol, GM2 and GM3 gangliosides, lactosylceramide, glucosylceramide, and lysobisphosphatidic acid. The accumulation of these substrates is thought to reflect impaired intracellular trafficking mediated by the NPC1 and NPC2 proteins respectively [[Watari et al 1999](#), [Liscum & Sturley et al 2004](#), [Mukherjee & Maxfield 2004](#)].

## *NPC2*

**Normal allelic variants:** *NPC2* has five [exons](#) and a single transcript of 0.9 kb in all tissues. It has been mapped to 14q24.3 [[Chikh et al 2004](#)].

**Pathologic allelic variants:** Two individuals were originally described with [mutations](#) in *NPC2* [[Naureckiene et al 2000](#)]. One individual was homozygous for c.58G>T in [exon](#) 1, and the other was a [compound heterozygote](#) for c.58G>T and c.332delA. A comprehensive study of eight families with *NPC2* found five [mutations](#) in the 16 mutant [alleles](#) identified (p.Glu20X, p.Glu118X, c.27delG, c.190\_5G>A, p.Ser67Pro) [[Millat et al 2001a](#)]. Except for c.27delG, the [mutations](#) were all homozygous. More recent studies have identified a total of 13 [disease-causing mutations](#), including five [missense mutations](#) and six that code for a premature stop [codon](#) [[Chikh et al 2005](#)].

Table 3. *NPC2* Pathologic Allelic Variants Discussed in This *GeneReview*

<a href="#">DNA Nucleotide</a> Change (Alias <sup>1</sup> )	Protein Amino Acid Change	Reference Sequence
c.58G>T	p.Glu20X	
c.115G>A	p.Val39Met	
c.133C>T	p.Gln45X	
c.141C>A	p.Cys47X	
c.199T>C	p.Ser67Pro	<a href="#">NM_006432.3NP_006423.1</a>
c.295T>C	p.Cys99Arg	
c.332delA	p.Asn111IlefsX5	
c.352G>T	p.Glu118X	
c.27delG	p.Alal2ArgfsX23	
c.190+5G>A (IVS2+5G>A) --		

See [Quick Reference](#) for an explanation of nomenclature. *GeneReviews* follows the standard naming conventions of the Human [Genome](#) Variation Society (<http://www.hgvs.org>).

1. Variant designation that does not conform to current naming conventions

**Normal [gene product](#):** The *NPC2* [gene product](#) is a 132-amino acid glycoprotein that is expressed in all tissues examined, with the highest

concentrations being found in epididymal fluid as well as in testis, kidney, and liver. NPC2 protein is soluble, binds cholesterol, and is able to partially reverse the lipid accumulation in NPC2 fibroblasts when added to the medium in culture. Added NPC2 has no effect on NPC1 fibroblasts in culture [[Naureckiene et al 2000](#)]. Different [isoforms](#) varying from 19 to 23 kd are distributed in a tissue-specific fashion, reflecting variable glycosylation [[Vanier & Millat 2004](#)]. Only the Asn58 residue needs to be glycosylated to ensure accurate targeting. NPC2 protein binds the mannose-6-phosphate receptor, and, in contrast, is not dependent on the presence of cholesterol for lysosomal targeting. NPC2 mainly colocalizes with LAMP1 but is also distributed to LAMP1-negative organelles [[Vanier & Millat 2004](#)].

## Resources

*See [Consumer Resources](#) for disease-specific and/or umbrella support organizations for this disorder. These organizations have been established for individuals and families to provide information, support, and contact with other affected individuals. GeneTests provides information about selected organizations and resources for the benefit of the reader; GeneTests is not responsible for information provided by other organizations.—ED.*

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Medical Genetic Searches: A specialized PubMed search designed for clinicians that is located on the PubMed Clinical Queries page. [PubMed](#)

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### **Published Statements and Policies Regarding Genetic Testing**

No specific guidelines regarding genetic testing for this disorder have been developed.

### **Suggested Reading**

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## Chapter Notes

## Author Notes

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## Revision History

- 22 July 2008 (me) Comprehensive update posted live
- 9 July 2007 (cd) Revision: [prenatal diagnosis](#) using biochemical testing no longer available clinically
- 13 February 2006 (me) Comprehensive update posted to live Web site
- 4 February 2004 (mp) Revision: testing
- 18 December 2003 (me) Comprehensive update posted to live Web site
- 10 September 2001 (mp) Revision
- 26 January 2000 (me) Review posted to live Web site
- 20 October 1999 (mp) Original submission

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Original Article

## Long-Term Miglustat Therapy in Children With Niemann-Pick Disease Type C

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Niemann-Pick disease type C is a rare, genetic disease associated with impaired intracellular lipid trafficking and progressive neurological symptoms. Miglustat slowed disease progression in a 12-month randomized trial in juveniles and adults with Niemann-Pick disease type C, and in a parallel, noncontrolled study in affected children. Here, the authors report the open-label extension to the pediatric study. Patients aged 4 to 12 years received open-label miglustat (dose adjusted for body surface area) for an initial 12 months, during a further 12-month extension, and a long-term, continued extension phase. Efficacy assessments included horizontal saccadic eye

movement, swallowing, and ambulation. Ten children completed 24 months' treatment. Horizontal saccadic eye movement, ambulation, and swallowing were stabilized at 24 months. Analysis of key parameters of disease progression showed disease stability in 8 of 10 patients (80%). Miglustat stabilized neurological disease progression in pediatric patients with Niemann-Pick disease type C, with comparable safety and tolerability to that observed in adults and juveniles.

**Keywords:** miglustat; Niemann-Pick disease type C; efficacy; safety

Niemann-Pick disease type C is a rare lysosomal storage disorder, estimated to occur in 1 in every 150 000 live births.<sup>1</sup> Niemann-Pick disease type C is inherited in autosomal recessive fashion and is attributed to mutations in the genes, *NPC1* (in 95% of cases)<sup>2,3</sup> and *NPC2* (in about 4% of cases).<sup>4,5</sup> *NPC1* and *NPC2*

gene mutations cause severe impairment of intracellular lipid transport, in turn, leading to the accumulation of unesterified cholesterol and other lipids in perinuclear lysosomes.<sup>6,7</sup> Unesterified cholesterol, sphingomyelin, phospholipids, and glycosphingolipids accumulate in the liver and spleen,<sup>8</sup> and glucosylceramide, lactosylceramide, and  $\text{GM}_2$  and  $\text{GM}_3$  gangliosides build up in the brain.<sup>8-10</sup> Foam cells (lipid-laden macrophages) accumulate in the viscera, lipid storage materials collect in neurons and glia,<sup>6</sup> and meganeurites, ectopic dendrites, and Alzheimer-like neurofibrillary tangles throughout the central nervous system are common neuropathological features.<sup>11</sup>

Niemann-Pick disease type C may present from infancy through adulthood but most commonly arises during middle-to-late childhood.<sup>12-14</sup> Early-onset (perinatal or early-infantile) disease is usually recognized on detection of unexplained cholestatic disease (eg, hepatosplenomegaly, prolonged jaundice, liver failure). Most infantile-onset cases present with hepatosplenomegaly<sup>15</sup>; thrombocytopenia, likely related to splenomegaly, is common. Late infantile-onset cases present with hypotonia and developmental delay, followed by dystonia, ataxia, dysphagia, and dysarthria.<sup>15-17</sup> Childhood-onset Niemann-Pick disease type C typically presents as ataxia, cognitive impairment, and clumsiness. Impaired vertical saccadic eye movements are invariably present but are often overlooked. Gelastic cataplexy and epileptic seizures are also common; progressive dystonia, dysphagia, and dysarthria manifest as

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# Long-Term Miglustat Therapy in Children With Niemann-Pick Disease Type C

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Niemann-Pick disease type C is a rare, genetic disease associated with impaired intracellular lipid trafficking and progressive neurological symptoms. Miglustat slowed disease progression in a 12-month randomized trial in juveniles and adults with Niemann-Pick disease type C, and in a parallel, noncontrolled study in affected children. Here, the authors report the open-label extension to the pediatric study. Patients aged 4 to 12 years received open-label miglustat (dose adjusted for body surface area) for an initial 12 months, during a further 12-month extension, and a long-term, continued extension phase. Efficacy assessments included horizontal saccadic eye

movement, swallowing, and ambulation. Ten children completed 24 months' treatment. Horizontal saccadic eye movement, ambulation, and swallowing were stabilized at 24 months. Analysis of key parameters of disease progression showed disease stability in 8 of 10 patients (80%). Miglustat stabilized neurological disease progression in pediatric patients with Niemann-Pick disease type C, with comparable safety and tolerability to that observed in adults and juveniles.

**Keywords:** miglustat; Niemann-Pick disease type C; efficacy; safety

**N**iemann-Pick disease type C is a rare lysosomal storage disorder, estimated to occur in 1 in every 150 000 live births.<sup>1</sup> Niemann-Pick disease type C is inherited in autosomal recessive fashion and is attributed to mutations in the genes, *NPC1* (in 95% of cases)<sup>2,3</sup> and *NPC2* (in about 4% of cases).<sup>4,5</sup> *NPC1* and *NPC2*

gene mutations cause severe impairment of intracellular lipid transport, in turn, leading to the accumulation of unesterified cholesterol and other lipids in perinuclear lysosomes.<sup>6,7</sup> Unesterified cholesterol, sphingomyelin, phospholipids, and glycosphingolipids accumulate in the liver and spleen,<sup>6</sup> and glucosylceramide, lactosylceramide, and  $G_{M2}$  and  $G_{M3}$  gangliosides build up in the brain.<sup>8-10</sup> Foam cells (lipid-laden macrophages) accumulate in the viscera, lipid storage materials collect in neurons and glia,<sup>6</sup> and meganeurites, ectopic dendrites, and Alzheimer-like neurofibrillary tangles throughout the central nervous system are common neuropathological features.<sup>11</sup>

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the disease advances.<sup>13,18-20</sup> Disease progression and symptomatology and overall life expectancy appear to be strongly influenced by the age at symptom onset. Children with early-onset disease tend to experience rapid symptomatic decline, with death often occurring before the age of 5 years.<sup>13</sup> Many patients with childhood-onset disease die before 20 years of age, although some survive into their 30s.<sup>6,13,17</sup>

There is no cure for Niemann-Pick disease type C, and until recently, only symptomatic therapies were available. Miglustat (Zavesca; Actelion Pharmaceuticals Ltd, Allschwil, Switzerland), a small aminosugar molecule that reversibly inhibits glucosylceramide synthase,<sup>21</sup> was recently approved for the treatment of progressive neurological manifestations in adult and pediatric patients in Europe. Miglustat was proposed as therapy for Niemann-Pick disease type C on the basis of beneficial effects seen in studies of murine and feline models of the disease.<sup>22</sup>

A randomized clinical trial with miglustat in juveniles and adults with Niemann-Pick disease type C suggested that miglustat stabilized neurological manifestations after 12 months<sup>23</sup>; similar effects were observed in children in a 12-month study conducted in parallel with this trial.<sup>24</sup> Here, we report efficacy data from pediatric patients who received miglustat for up to 24 months and long-term safety and tolerability data from extension therapy up to 52 months. Findings from an analysis of key markers of neurological disease progression in patients treated for at least 12 months are also reported to assess the effect of miglustat on disease stability.

## Methods

### Patients/Design

This study consisted of an initial 12-month, open-label, noncontrolled treatment period<sup>23</sup> plus a prospective, 12-month, open-label extended phase followed by a continued extension phase. Children with Niemann-Pick disease type C aged between 4 and 11 years, with a diagnosis confirmed by a typical pattern of filipin staining and impaired cholesterol esterification in cultured fibroblasts, and who were able to ingest medication in capsule form, were eligible for this study. Excluded criteria included clinically significant diarrhea (>3 liquid stools per day for >7 days) without definable cause within 3 months of the screening visit, a history of significant gastrointestinal disorders, or an adjusted creatinine clearance <70 mL/min per 1.73 m<sup>2</sup>. Patients were permitted to receive other concomitant medications that were deemed necessary for managing their disease.

All children received miglustat throughout all periods of the study. The pediatric dose was adjusted from the adult starting dose of miglustat (200 mg orally thrice daily) according to patient body surface area using the following equation:  $(\text{body surface area, m}^2/1.8) \times 200$ . Dose reduction guidelines were provided for patients who experienced unacceptable adverse events considered related to miglustat. All patients' tolerance

of therapy was assessed approximately 1 week after commencing miglustat and monthly thereafter.

This study was performed under an Investigational New Drug application and complied with US Food and Drug Administration regulations. It was conducted in accordance with the Declaration of Helsinki (1964) and later amendments. Written informed consent was obtained from a parent or legal guardian for all patients.

### Assessments

The primary efficacy end point was the change in main-sequence horizontal saccadic eye movement asymptotic peak velocity (HSEM- $\alpha$ ) from baseline to month 12 and month 24. Measurements of saccadic eye movement were performed as described previously.<sup>23</sup> On each occasion, 2 saccadic eye movement evaluations were performed within 24 hours of each other, separated by at least 1 hour. Data were sent to a central assessor for blinded assessment of main-sequence horizontal saccadic eye movement asymptotic peak velocity. For the initial 12-month study, data from sequential visits were randomized for each patient so that analysis was undertaken not knowing which data originated from which visit. Month 24 assessments were reviewed in an unblinded fashion.

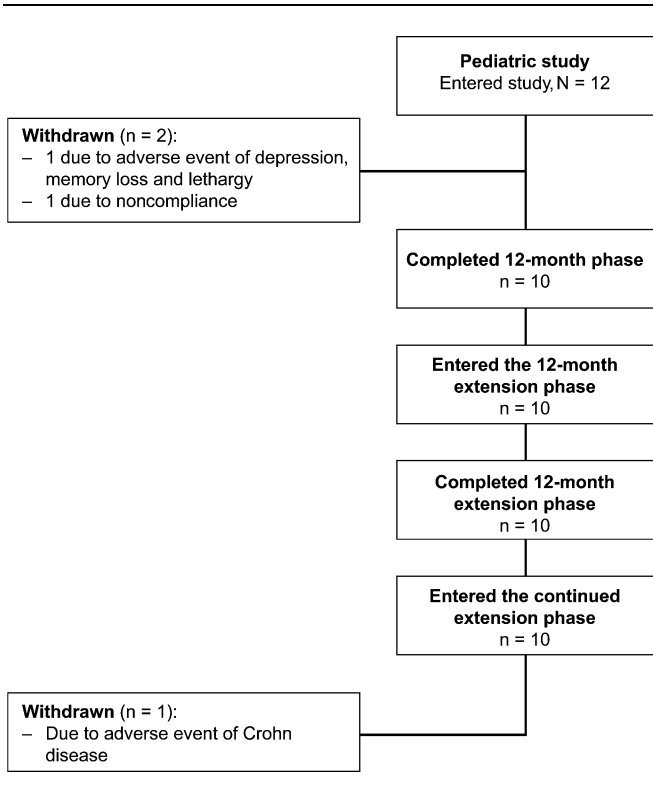
Secondary end points included change from baseline to month 12 and month 24 in main-sequence horizontal saccadic eye movement initial peak velocity-amplitude slope (HSEM- $\beta$ ) and vertical saccadic eye movement parameters; neurological and neuropsychological assessments; assessment of swallowing (based on a patient's ability to swallow various substances: water, puree, soft lumps, and one third of a cookie); and ambulation (as measured by the Standard Ambulation Index). Standard Ambulation Index scores range from 0 to 9, with a lower score indicating better ambulation.

Safety assessments were conducted throughout the study (up to 52 months) and included monitoring of adverse events, routine safety laboratory tests (including liver function tests, and hemoglobin and platelet counts), standard neurological evaluation, physical examination (including muscle strength and reflexes), vital signs, height, and weight. Concomitant medications were recorded at every postscreening visit.

### Data Analysis

The efficacy population included all patients who completed 24 months of miglustat treatment (the initial 12-month phase and the 12-month extended phase). The results of primary and secondary efficacy evaluations are presented for all patients who completed 24 months of miglustat treatment. The safety set included all patients who completed 24 months of miglustat treatment and who entered the continued extension phase.

Data were evaluated using descriptive methods; no statistical testing was applied. For primary and secondary efficacy parameters (horizontal saccadic eye movement asymptotic peak velocity and Standard Ambulation Index), data were summarized using mean and 95% confidence intervals at baseline, month 12, and month 24. For swallowing, the percentage of patients improved or stable at month 24 was calculated. Safety and tolerability data are also reported using descriptive parameters.



**Figure 1.** Patient disposition.

A descriptive disease stability analysis of key parameters of neurological disease (horizontal saccadic eye movement asymptotic peak velocity, swallowing, ambulation) was performed in patients completing at least 12 months of treatment. Patients were classified as having “stable disease” if there was no deterioration in swallowing or ambulation or if only horizontal saccadic eye movement asymptotic peak velocity deteriorated. “Deterioration” was defined for each parameter as follows: horizontal saccadic eye movement asymptotic peak velocity, an increase of >20% from baseline; swallowing function, any downgrading versus baseline; and ambulation, an increase of >1 point from baseline.

## Results

### Patients and Treatment

A total of 12 children were enrolled into this study at 2 centers in the United Kingdom and the United States, between August 2003 and September 2004. The entire study period was August 11, 2003 (first patient, first visit), to January 29, 2008 (last patient, last visit). In the initial 12-month phase of the study, 2 patients withdrew from the study prematurely.<sup>23</sup> All 10 patients who completed the initial 12-month phase entered the 12-month extended phase, and no further patients withdrew from the study during this period. All 12 patients who initially enrolled received at least 1 dose of study medication (Figure 1).

**Table 1.** Demographics and Baseline Patient Characteristics

	All Patients (N = 12)
Gender	
Male, n (%)	5 (42)
Female, n (%)	7 (58)
Age (years)	
Mean $\pm$ SD	7.2 $\pm$ 2.5
Median	7.0
Range	4-11
Weight (kg)	
Mean $\pm$ SD	27.9 $\pm$ 10.6
Median	25.9
Range	13.2-43.8
Height (cm)	
Mean $\pm$ SD	124.3 $\pm$ 19.9
Median	123.0
Range	95-160
Body surface area (cm <sup>2</sup> )	
Mean $\pm$ SD	9728 $\pm$ 2677
Median	9352
Range	6018-14 093

Note: SD, standard deviation.

**Table 2.** Baseline Disease Manifestations

	Number (%) of Patients (N = 12)
Number of patients with $\geq 1$ manifestation of NP-C	12 (100)
Vertical supranuclear gaze palsy	12 (100)
Ataxia	10 (83)
Splenomegaly	10 (83)
Cognitive impairment	8 (67)
Speech	7 (58)
Hepatomegaly	7 (58)
Difficulty in positioning of limbs	5 (42)
Pyramidal tract dysfunction	5 (42)
Swallowing difficulties	4 (33)
Cataplexy	4 (33)
Seizures	0
Other	11 (92)

Note: NP-C, Niemann-Pick disease type C.

The main demographics and baseline characteristics of all enrolled patients are summarized in Table 1. The mean age of the 10 patients who entered the continued extension phase was 7.2 (range, 4-11) years. The most frequently occurring manifestations in Niemann-Pick disease type C were vertical supranuclear gaze palsy (12 patients, 100%), ataxia (10 patients, 83%), splenomegaly (10 patients, 83%), and cognitive impairment (8 patients, 67%; Table 2).

Over the entire study period, the median (range) exposure to miglustat was 1073 (725-1604) days; the median (range) dose was 350 (100-600) mg/d. Only 2 patients required dose modification over the study period. One patient had his dose reduced from 200 mg twice daily to

**Table 3.** Horizontal Saccadic Eye Movement Values and Standard Ambulation Index Scores at Baseline, Month 12, and Month 24

	n <sup>a</sup>	Mean	95% CI
HSEM- $\alpha$ (ms/deg) <sup>b</sup>			
Baseline	9	2.181	1.3-3.0
Month 12	9	1.692	1.0-2.4
Month 24	9	2.106	1.3-2.9
HSEM- $\beta$ (ms/deg) <sup>b</sup>			
Baseline	9	28.96	13.9-44.0
Month 12	9	33.66	18.3-49.0
Month 24	9	33.47	17.9-49.1
Standard Ambulation Index scores <sup>c</sup>			
Baseline	10	2.0	0.7-3.3
Month 12	10	2.3	0.6-4.0
Month 24	10	2.6	0.7-4.5

Note: CI, confidence interval; HSEM- $\alpha$ , horizontal saccadic eye movement asymptotic peak velocity; HSEM- $\beta$ , horizontal saccadic eye movement initial peak velocity-amplitude slope.

a. No horizontal saccadic eye movement data available from 1 patient.

b. A decrease in horizontal saccadic eye movement value indicates improvement.

c. Lower Standard Ambulation Index scores indicate better ambulation.

100 mg thrice daily after 4 months due to an adverse event of moderate, intermittent diarrhea, and subsequently withdrew from the study in the initial 12-month phase. The other patient had her original dose of 200 mg twice daily reduced to 300 mg once daily after 10 months in an attempt to reduce tremors and dysarthria. The patient remained on this dose and completed the study.

## Efficacy

**Horizontal saccadic eye movement.** Mean (95% confidence interval) horizontal saccadic eye movement asymptotic peak velocity values at baseline, month 12, and month 24 are summarized in Table 3. A mean (95% confidence interval) decrease in horizontal saccadic eye movement asymptotic peak velocity from 2.181 (1.3, 3.0) at baseline to 1.692 (1.0, 2.4) at month 12 and 2.106 (1.3, 2.9) at month 24 was noted. Mean horizontal saccadic eye movement initial peak velocity-amplitude slope values increased (ie, worsened) from baseline to month 12, but did not deteriorate further to month 24 (Table 3).

**Ambulation.** Mean (95% confidence interval) Standard Ambulation Index scores showed a small increase from 2.0 (0.7, 3.3) at baseline to 2.3 (0.6, 4.0) at month 12 and 2.6 (0.7, 4.5) at month 24 (Table 3).

**Swallowing.** Most of the patients were able to swallow all 4 substances (water, puree, soft lumps, and one third of a cookie) easily at baseline, and this was sustained at month 24. Changes in swallowing ability over the course of the

study were seen in tests with one third of a cookie. Eight patients (80%) had no change in ability to swallow one third of a cookie (all were able to swallow easily at baseline and month 24), while 1 patient improved (10%) and 1 deteriorated (10%).

**Disease stability analysis.** Eight patients (80%) were categorized as having stable disease, and 2 patients (20%) were categorized as deteriorating during the study. Horizontal saccadic eye movement asymptotic peak velocity was improved or stable in 6 of 9 (67%) evaluable patients. Nine patients (90%) had normal swallowing function at both baseline and month 24. Swallowing function was mildly impaired at baseline in 1 patient, which deteriorated at month 24. Ambulation was categorized as stable at month 24 in 8 patients (80%).

## Safety and Tolerability

The majority of adverse events were mild or moderate in severity. Two patients discontinued treatment due to adverse events: 1 patient in the initial 12-month phase because of depression, memory loss, and lethargy and 1 patient in the continued extension phase because of Crohn disease.

Diarrhea was the most common adverse event (8 patients, 67%) and tremor was the most common non-gastrointestinal adverse event (7 patients, 58%; Table 4). Most occurrences of gastrointestinal adverse events (diarrhea, flatulence, abdominal pain) were considered related to miglustat therapy. Most other adverse events were considered unrelated to miglustat.

The period prevalence of most adverse events was either stable or decreased over time. Diarrhea was reported in 6 of 10 patients (60%) at 1 year, 6 of 10 patients (60%) at 2 years, and 4 of 7 patients (57.1%) at 3 years. Adverse events for which period prevalence increased were mostly related to nervous system disorders (supranuclear palsy, tremor, dystonia, and dementia); tremor was reported in 2 of 10 patients (20%) at 1 year, 2 of 10 patients (20%) at 2 years, and 3 of 7 patients (42.9%) at 3 years. Most nervous system events represent typical manifestations of Niemann-Pick disease type C and were considered unrelated to miglustat. Weight decrease, considered related to miglustat, was recorded as an adverse event in 3 patients overall (25%). The prevalence of this adverse event decreased from 3 of 10 patients (30%) at 1 year to 2 of 10 patients (20%) at 2 years and 1 of 7 patients (14.3%) at 3 years. No clinically relevant effect on growth curves was observed.

Five patients (50%) reported 10 serious adverse events, all of which were considered unrelated to miglustat. One patient reported 5 serious adverse events: Crohn disease, diarrhea, painful defecation, perineal abscess, and acrochordons (skin tags). Another patient reported severe

**Table 4.** Incidence of Treatment-Emergent Adverse Events<sup>a</sup> From Baseline to End of Study (up to 52 Months; Safety Population, n = 12)

Adverse Event	Incidence	
	n	%
Diarrhea	8	66.7
Tremor	7	58.3
Headache	6	50.0
Cough	5	41.7
Fatigue	5	41.7
Gait disturbance	5	41.7
Vomiting	5	41.7
Ataxia	4	33.3
Dysphagia	4	33.3
Dystonia	4	33.3
Flatulence	4	33.3
Gaze palsy	4	33.3
Nasopharyngitis	4	33.3
Splenomegaly	4	33.3
Cataplexy	3	25.0
Constipation	3	25.0
Deafness	3	25.0
Dementia	3	25.0
Dysarthria	3	25.0
Epistaxis	3	25.0
Fall	3	25.0
Hyperreflexia	3	25.0
Lethargy	3	25.0
Pyramidal tract syndrome	3	25.0
Respiratory tract infection	3	25.0
Sinusitis	3	25.0
Weight decrease	3	25.0

a. Occurring in  $\geq 20\%$  of patients, overall.

dehydration and vomiting. Of the remaining 3 patients, 1 reported abnormal gait, 1 severe cellulitis, and 1 respiratory syncytial virus infection.

There was no pattern of clinically significant abnormal laboratory findings. Mean (standard deviation) platelet count decreased from baseline by  $-34.0$  ( $52.9$ )  $10^9/L$ , but this was not considered clinically relevant, and no patient had a platelet count  $<120 \times 10^9/L$ . Most patients had biochemistry values within normal ranges, and mean changes were usually small and not clinically relevant. Mean (standard deviation) aspartate transaminase activity decreased by  $11.8$  ( $13.6$ ) U/L (approximately 17%). Mean (standard deviation) alanine transaminase activity fell by  $-0.3$  ( $8.5$ ) U/L (1.5%).

## Discussion

While Niemann-Pick disease type C may present clinically at any age, the late-infantile and juvenile forms are the most common presentations, representing 60% to 70% of cases. Our data suggest that the apparent effect of miglustat on disease stability observed in children during

the initial 12 months of treatment<sup>23</sup> was sustained at 24 months. Horizontal saccadic eye movement asymptotic peak velocity, ambulation, and swallowing all appeared stable at 24 months. There was little scope for improvement in swallowing ability, as the majority of patients could easily swallow all 4 test substances at baseline. Analysis of individual patient records for key neurological disease parameters (swallowing and Standard Ambulation Index) indicated overall stabilization in 80% of patients. Stabilization was therefore considered the best possible outcome in most patients.

These data support findings from clinical experience with miglustat in children with Niemann-Pick disease type C. In a report of 2 cases from Taiwan, a 14-year-old child with severe difficulties in swallowing and ambulation experienced substantial improvements in swallowing and ambulation after 6 months of miglustat treatment.<sup>25</sup> A dramatic and sustained improvement in ambulation and improved swallowing were also reported in a 10-year-old Brazilian child after 12 months on miglustat.<sup>26</sup>

The safety/tolerability of miglustat in children was consistent with that observed in adult and juvenile Niemann-Pick disease type C patients.<sup>23</sup> Diarrhea and tremor were reported most frequently, and gastrointestinal adverse events (diarrhea, flatulence, abdominal pain) were most often considered related to treatment. These adverse events were treatable with dietary modification (eg, avoiding high lactose- or carbohydrate-content foods) and/or antipropulsive medications. Miglustat had no clinically relevant impact on growth curves, despite some mild and transient effects on body weight and body mass index.

The noncontrolled design of this study inevitably limits the interpretation of these data. Our observations contrast with the steady decline that is reported in children with Niemann-Pick disease type C who are not treated with miglustat,<sup>15,17,27</sup> suggesting that the stabilization of key disease parameters seen here represents a therapeutic effect. Further data from longitudinal studies of disease progression among children are required to help further define the treatment effect of miglustat over the long term.

Although ocular motor measures provide an index of disease severity in Niemann-Pick disease type C and may be a useful adjunct for monitoring the illness progress and medication response,<sup>28,29</sup> the sensitivity of horizontal saccadic eye movement asymptotic peak velocity as a clinical efficacy measure is still under debate, as it has not been formally correlated with survival or specific neurologic functions.

## Conclusion

This study indicates that miglustat may stabilize neurological disease in children with Niemann-Pick disease type C and that it is well tolerated. Disease stabilization is

considered the best attainable therapeutic goal in Niemann-Pick disease type C because of the irreversible neuronal damage that is likely to be present by the time patients are diagnosed; improvements should not generally be expected.

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## Niemann-Pick C1 Disease: Correlations between *NPC1* Mutations, Levels of NPC1 Protein, and Phenotypes Emphasize the Functional Significance of the Putative Sterol-Sensing Domain and of the Cysteine-Rich Luminal Loop

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To obtain more information of the functional domains of the NPC1 protein, the mutational spectrum and the level of immunoreactive protein were investigated in skin fibroblasts from 30 unrelated patients with Niemann-Pick C1 disease. Nine of them were characterized by mild alterations of cellular cholesterol transport (the "variant" biochemical phenotype). The mutations showed a wide distribution to nearly all NPC1 domains, with a cluster (11/32) in a conserved NPC1 cysteine-rich luminal loop. Homozygous mutations in 14 patients and a phenotypically defined allele, combined with a new mutation, in a further 10 patients allowed genotype/phenotype correlations. Premature-termination-codon mutations, the three missense mutations in the sterol-sensing domain (SSD), and A1054T in the cysteine-rich luminal loop all occurred in patients with infantile neurological onset and "classic" (severe) cholesterol-trafficking alterations. By western blot, NPC1 protein was undetectable in the SSD missense mutations studied (L724P and Q775P) and essentially was absent in the A1054T missense allele. Our results thus enhance the functional significance of the SSD and demonstrate a correlation between the absence of NPC1 protein and the most severe neurological form. In the remaining missense mutations studied, corresponding to other disease presentations (including two adults with nonneurological disease), NPC1 protein was present in significant amounts of normal size, without clear-cut correlation with either the clinical phenotype or the "classic"/"variant" biochemical phenotype. Missense mutations in the cysteine-rich luminal loop resulted in a wide array of clinical and biochemical phenotypes. Remarkably, all five mutant alleles (I943M, V950M, G986S, G992R, and the recurrent P1007A) definitively correlated with the "variant" phenotype clustered within this loop, providing new insight on the functional complexity of the latter domain.

### Introduction

Niemann-Pick type C (NPC [MIM 257220]) disease is a neurodegenerative lysosomal lipid storage disease, with autosomal recessive transmission, characterized by lysosomal/late-endosomal accumulation of endocytosed unesterified cholesterol (Pentchev et al. 1995; Patterson et al. 2001). The clinical manifestations of NPC are heterogeneous. Most patients have a progressive neurologic disease, but both age at onset, which ranges from early

infancy to adulthood, and subsequent course vary (Vanier and Suzuki 1996; Patterson et al. 2001). We have also documented a wide variation in severity of the cellular cholesterol lesion, with typical severe alterations described as the "classic" biochemical phenotype and mild alterations as the "variant" phenotype (Vanier et al. 1991b).

Although the exact location and nature of the trafficking defect(s) are still under investigation (Liscum and Munn 1999; Blanchette-Mackie 2000; Cruz and Chang 2000; Davies et al. 2000; Ory 2000), substantial advances have occurred in our knowledge of NPC. Cell-hybridization studies and linkage analysis (Steinberg et al. 1994; Vanier et al. 1996) demonstrated the existence of two complementation groups. *NPC1*, the disease-causing gene in >95% of NPC patients, has been located to 18q11-q12 and is now fully characterized (Carstea

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et al. 1997; Morris et al. 1999). Very recently, a previously identified gene, *HE1*, mapped to chromosome 14q24.3, was recognized as the gene mutated in the minor NPC2 complementation group (Naureckiene et al. 2000). The finding of identical clinical, cellular, and biochemical phenotypes in patients belonging to the two groups (Vanier et al. 1996; Christomanou et al. 2000) led to the conclusion that both gene products may function in tandem or sequentially (Vanier et al. 1996; Carstea et al. 1997).

The *NPC1* cDNA sequence (see Genbank) predicts a protein of 1,278 amino acids. Topological analysis of NPC1 has revealed that the protein contains 13 transmembrane domains, three large and four small luminal loops, six small cytoplasmic loops, and a cytoplasmic tail (Davies and Ioannou 2000). The region located between amino acid residues 615 and 797 shows strong homology to the sterol-sensing domain (SSD) identified in several other integral membrane proteins that respond to endoplasmic reticulum cholesterol (Carstea et al. 1997). This putative SSD has the same orientation as those in HMG-CoA reductase and SCAP (sterol regulatory element binding protein [SREBP] cleavage activation protein) (Davies and Ioannou 2000) and appears to have important functional significance (Watari et al. 1999). The mature protein appears glycosylated, with a size of 170–190 kilodaltons (kD) (Higgins et al. 1999; Watari et al. 1999), and there is convincing evidence that NPC1 resides in late endosomes and interacts transiently with lysosomes and the trans-Golgi network (Higgins et al. 1999; Neufeld et al. 1999). A number of studies point toward a key role for the NPC1 protein in modulating vesicular trafficking of cholesterol and of glycolipids (Neufeld et al. 1999; Blanchette-Mackie 2000; Ory 2000; Zhang et al. 2001), but recent data suggest that NPC1 is a permease acting as a transmembrane efflux pump (Davies et al. 2000).

There are still only limited published studies on NPC1 mutations (Carstea et al. 1997; Greer et al. 1998, 1999; Millat et al. 1999; Yamamoto et al. 1999, 2000). The mutation prevalent in patients with Acadian Nova Scotian origins (Niemann-Pick type D) was identified as G992W (Greer et al. 1998), and that prevalent in Hispanic patients from the Upper Rio Grande area of the United States was identified as I1061T (Millat et al. 1999). The latter allele was further shown to be the most common NPC1 mutation among individuals of western European descent (Millat et al. 1999). Greer et al. (1999) pointed out that a majority of mutations were clustered in a luminal cysteine-rich loop, highlighting the potential functional importance of this domain. On the other hand, the study by Yamamoto et al. (2000), including mutational and immunoblotting studies, as well as information on the clinical presentation, disclosed a much wider distribution of the mutations. Pre-

liminary results from the present study were presented in abstract form (Vanier et al. 1999; Vanier and Millat 2000).

Identification of mutations and studies of the mutated protein in cultured skin fibroblasts of selected patients with mutations in NPC1 was initiated primarily to obtain more information on functional domains of the NPC1 protein. Although we attempted to cover the entire spectrum of clinical and biochemical phenotypes, we were particularly interested in studying two subsets of patients. Children with infantile neurological onset invariably showed pronounced cellular cholesterol abnormalities (Vanier et al. 1988, 1991a); they clearly corresponded to the most severe form of the disease and therefore were best suited to pinpoint the most critical regions of the protein. Among patients with other clinical phenotypes, a majority share the same “classic” biochemical pattern, but about one-fifth show moderate alterations of cellular cholesterol trafficking, described as the “variant phenotype” (Vanier et al. 1991b). The biochemical subtype has been shown to be constant within a sibship (Vanier et al. 1991b) and thus is most likely defined by the genotype. Whether the variant phenotype is correlated with alterations of a specific domain of the protein is not yet known. To address this question, we have studied patients with late onset showing either severe or mild cholesterol-trafficking abnormalities. Since no biochemical marker has been found, so far, to correlate with a given clinical phenotype, the second aim of this study has been to evaluate correlations between genotype and clinical phenotype. Overall, our findings allow assignment of existing or new allelic combinations to specific phenotypes and provide new information on the functional significance of two particular domains of the NPC1 protein.

## Subjects and Methods

### Subjects

Cultured skin fibroblasts were obtained from 30 unrelated NPC patients from various ethnic backgrounds, including French (13), German (4), British (4), Dutch (1), Gypsy (1), Tunisian (3), Turkish (2), Pakistani (1), and African American (1). Close consanguinity was certain or likely in 10 families. The methods used for diagnosis by evaluation of cellular cholesterol by filipin staining and of LDL-induced cholesteryl ester formation were those described by Vanier et al. (1991b). Genetic-complementation analysis was performed prior to molecular studies to ensure that all patients belonged to the main (NPC1) complementation group (Vanier et al. 1996). Classification of patients with respect to their clinical and biochemical characteristics (table 1) was as proposed by us elsewhere (Vanier et al. 1988, 1991a,

Table 1

## Clinical Summary of the 30 Families Included in the Study

Family	Cell Line Code	Biochemical Phenotype <sup>a</sup>	Clinical Phenotype <sup>b</sup>	Age at Death (years)	Age at Last Follow-up (years)	Ethnicity	Consanguinity	Parents Available	Reference
1	98119	Classic	Infantile		2.83	African American	No	No	Dawson et al. 1971
2	80001	Classic	A: Infantile	3.83		Gypsy	Yes	No	Vanier et al. 1988, case 2
			B: Infantile	3.33					
			C: Cholestatic	.30					
3	87024	Classic	A: Infantile	2.58		Tunisian	Yes	Yes	Kanoun et al. 1989
			B: Cholestatic	.25					
4	91029	Classic	A: Infantile	5.16		French	Yes	Yes	
			B: Infantile	4.92					
5	81057	Classic	A: Cholestatic	.04		French	Yes	No	Vanier et al. 1988, case 10
			B: Infantile	3.16					
6	83024	Classic	Infantile	5.08		Turkish	Likely	No	
7	83049	Classic	Infantile	5.33		French/West Indies	No	No	Vanier et al. 1988, case 13
8	82052	Classic	Infantile	3.5		French	No	No	Vanier et al. 1988, case 8 Vanier et al. 1999
9	79011	Classic	Infantile	5.0		Tunisian	No	No	Vanier et al. 1988, case 14
10	96115	Classic	Severe late infantile		4.50	French/West Indies	No	Yes	
11	82042	Classic	Late infantile	5.75		French	No	No	Vanier et al. 1988, case 16
12	95085	Classic	A: No neurological signs (cardiac problem)	1.00		Pakistani	Yes	No	
			B: Late infantile		6.00				
			C: Late infantile		5.75				
13	90104	Variant	Late Infantile	10		German	No	Yes	
14	93094	Classic	A: Late infantile	11.66		French	No	Yes	Millat et al. 1999, family 8
			B: Late infantile	8.00					
15	91048	Classic	A: Juvenile		24	French	No	Yes	Millat et al. 1999, family 1
			B: Juvenile		20				
			C: Juvenile		20				
			D: Juvenile						
16	98009	Classic	Juvenile	16	32	French	No	No	Millat et al. 1999, family 15
17	90096	Variant	A: Juvenile	18		British	No	Yes	
			B: Juvenile		24				
18	94139	Variant	Juvenile		25	British	No	No	
19	92114	Variant	A: Juvenile		28	German	No	No	
			B: Juvenile		22				
20	95070	Variant	Juvenile		13	British	No	No	
21	97089	Variant	Juvenile		20	Dutch	Yes	Yes	
22	96017	Variant	Adult		43.5	French	No	Yes	
23	96157	Classic	A: Adult	37		German	No	No	
			B: Adult		41				
24	90002	Variant	Adult without neurological symptoms (simple psychic structure)		66	German	Yes	No	Fröhlich et al. 1990
25	97014	Classic	Adult without neurological symptoms		50	British	No	No	Fensom et al. 1999
26	96084	Classic	No neurological symptoms		7.5	French	No	Yes	
27	91144	Variant	No neurological symptoms		9.0	French	No	Yes	
28	86065	Classic	Cholestatic	1.08		Tunisian	Yes	No	Vanier et al. 1988, case 39
29	97114	Classic	Cholestatic	.25		Turkish	Yes		Baumkötter et al. 1998
30	97119	Classic	Cholestatic	.5		French	No	No	

NOTE.—Families 1–25 are listed in order of decreasing severity of neurological phenotypes. Patients 26–30 could not be classified neurologically (too limited follow-up or early death resulting from liver failure).

<sup>a</sup> Defined by the degree of severity of alterations of intracellular cholesterol processing (Vanier et al. 1991b).

<sup>b</sup> Classification of clinical phenotypes by age at onset of neurological symptoms (except for the cholestatic rapidly fatal form) (Vanier and Suzuki 1996).

**Table 2****Sequence of Primers Used for RT-PCR**

Primer	Sequence (5'→3')	cDNA Coordinates <sup>a</sup>
NPC-As	GAG CCC AAC CAG CCG AAC	–51/–34
NPC-Aas	CCG AAC ATC ACA ACA GAG ACT GAC	234/211
NPC-Bs	CCA TTG CCA AAG GAT GGA TAT G	148/169
NPC-Bas	GCC TTG TCA TTA CTT GAG GGG G	515/494
NPC-Cs	CTA CGT CGG ACA GAG TTT TGC C	438/459
NPC-Cas	GCT ACA TGG TGC TGT GAC CTC ATC	717/694
NPC-Ds	CAA TGG ACA GGC ACC TTT TAC C	591/612
NPC-Das	CCG TTT TCT GTA GCA CCA CAC TG	888/866
NPC-Es	GCT TGG ACG CCA TGT ATG TCA TC	791/813
NPC-Eas	CCT GAC GAA CAC GCA GTA ATG AAG	1,100/1,077
NPC-Fs	CAG CAT TTG AGG GCT GCT TG	986/1,005
NPC-Fas	AAG CGG AGG TCC AAA GGG TAC ATC	1,305/1,282
NPC-Gs	CAA CCA ATC CAG TTG ACC TCT GG	1,121/1,143
NPC-Gas	AGC ACG GAA TGG CTG TTC TG	1,484/1,465
NPC-Hs	CAC GAA CTG CAC CAT TTT GAG TG	1,428/1,450
NPC-Has	TTG ACA GGG AAG GTA ATC ACA AGG	1,703/1,680
NPC-Is	TGT GTT GGG AGG CTA TGA TGA TC	1,635/1,657
NPC-Ias	CCT GCG ACA GCT TTT GAT GTG	1,941/1,921
NPC-Js	GTG ACA GTG ATG TCT TCA CCG TTG	1,850/1,873
NPC-Jas	AGG ATG ACA GGA ACA TAC TGG GAG	2,218/2,195
NPC-Ks	CCA GAG AGA TGA ACG TCT TCA AGG	2,127/2,150
NPC-Kas	CCC CAA GAG ACT CAC GAA ACA G	2,352/2,331
NPC-Ls	CGG GAT TGG CAG TCT TCA TTG	2,291/2,311
NPC-Las	TCC ACC ATG TAG GAG TCA TCT GG	2,621/2,599
NPC-Ms	AGC ATC GCA GTC CTG AAC AAA G	2,545/2,566
NPC-Mas	GAT ATT GTC CAC TCG ACA GCA AGA C	2,886/2,862
NPC-Ns	TTT CGA CTG GGT GAA GCC ACA G	2,838/2,859
NPC-Nas	GCG TCA ATA AAG TCA GCA GAG GTC	3,161/3,138
NPC-Os	AAG TGT GGC AAA GGG GGA CAT G	3,028/3,049
NPC-Oas	ACC GAG GTT GAA GAT AGT GTC GTC	3,309/3,286
NPC-Ps	ATA GCC AGT AAT GTC ACC GAA ACC	3,181/3,204
NPC-Pas	TTC ATG CTC ACC GTG AAC GCT C	3,539/3,518
NPC-Qs	AAC CTG GTG ATG AGC TGT GGC ATC	3,466/3,489
NPC-Qas	GTA TCG CTC TTC AGT GGC ACA AC	3,801/3,779
NPC-Rs	CTC CGT GTT CAG TGG AAT CAC AC	3,588/3,610
NPC-Ras	ACA CAG TTC AGT CAG GAT GCC C	3,869/3,848

<sup>a</sup> Counted from the adenosine residue of the initiation codon.

1991b; Vanier and Suzuki 1996; Millat et al. 1999). In summary, patients with neurological symptoms were categorized, by type and age at onset of first neurological symptoms, as having either a severe infantile form (onset at age <2 years), a late infantile form (onset at age 3–5 years), a juvenile form (onset at age >5–16 years), or an adult form (onset at age >16 years). The denomination “rapidly fatal cholestatic form” was applied to the disease of patients who died from liver failure in the first months of life. On the basis of the severity of impairment in intracellular cholesterol processing in cultured fibroblasts (Vanier et al. 1991b), patients were also classified into either a classic or a variant biochemical phenotype. The classic phenotype refers to patients with a striking accumulation of free cholesterol in lysosomes assessed by filipin staining, together with a severe block in LDL-induced cholesteryl ester formation. In cells from biochemically variant patients, cholesterol storage may be

evidenced only after challenge with pure LDL, and the rate of cholesteryl ester formation may not be affected. Cultured skin fibroblasts or peripheral blood samples were also available from the parents of 11 patients. Genomic DNA was obtained from a control population of 95 unrelated unaffected subjects and from 30 additional patients with mutations in NPC1, of whom 24 showed a variant biochemical phenotype.

#### DNA and RNA Isolation

Genomic DNA was isolated from skin fibroblasts or from whole blood, according to the protocol of Jeanpierre et al. (1987). Total RNA was extracted from fibroblasts monolayers using the Trizol reagent (Gibco BRL).

### Reverse-Transcription and SSCP Analysis

Reverse transcription was performed using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. NPC1 cDNA was amplified in 18 overlapping fragments, using primers described in table 2. Each PCR was performed in a 20- $\mu$ l reaction volume containing 0.4  $\mu$ l of cDNA, 200  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub>, PCR buffer, 1 U of EurobioTaq, 4 pmol of each primer, 0.1  $\mu$ l  $\alpha$ [<sup>33</sup>P]dATP, and 3,000 Ci/mmol (Amersham Pharmacia Biotech). Two microliters of the radiolabeled PCR product were mixed with 15  $\mu$ l stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol) and denatured for 5 min at 95°C. The sample was loaded on 0.5  $\times$  MDE gels (BioWhittaker Molecular Applications), with or without 10% glycerol. Electrophoresis was performed overnight in 0.6  $\times$  TBE running buffer, either at 20 W at 4°C or at 6W at room temperature.

### Mutation Analysis

Sequences of PCR products with aberrant SSCP patterns were determined on cDNA and on genomic DNA using the Thermosequencing cycle sequencing kit, with primers labeled with  $\gamma$ [<sup>33</sup>P]dATP, 3,000 Ci/mmol (Amersham Pharmacia Biotech). Because of the numerous known polymorphisms of the NPC1 gene (Morris et al. 1999; Yamamoto et al. 1999, 2000), for each new point mutation identified, genomic DNA from the patient and from 95 control samples was amplified using specific primers. PCR products were laid as a dot-blot on a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Membranes were hybridized with either wild-type or mutated  $\gamma$ [<sup>33</sup>P]dATP labeled oligoprobes. Whenever possible (table 1), the mutation was also studied in the parents.

### Detection of the P1007A Mutation by Introduction of an NheI Restriction Site

Genomic DNA was amplified using the sense primer 5'-TTGACCCTGCCTGCGTTC-3' and the antisense primer 5'-CTTTGCCACACTTGGGGCT-3'. The 40 PCR cycles each included steps of 45 s at 92°C for denaturation, at 60°C for annealing, and at 72°C for extension. The 126-bp PCR product was digested for 2 h at 37°C by NheI (Roche Diagnostics) and was analyzed on a 12% polyacrylamide gel. The mutation gave rise to a 105-bp product.

### Western Blot

Western blotting was performed as described by Yamamoto et al. (2000). Pellets from confluent fibroblast cultures (in MEM or DMEM, supplemented with 10% fetal calf serum) were homogenized and briefly sonicated

on ice in buffer A (50 mM Tris (pH8.0), 150 mM NaCl, 100  $\mu$ g/ml PMSF, and 1  $\mu$ g/ml aprotinin). The lysates were centrifuged for 20 min at 100,000 rpm (+4°C). After centrifugation, the supernatants were discarded. The pellets were sonicated again with buffer A containing 0.4% SDS and were centrifuged for 20 min at 13,000 rpm. The protein content of the supernatants was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce). The lysates (10  $\mu$ g protein) were subjected to 7.5% SDS/PAGE. After transfer overnight to nitrocellulose membranes (BioRad), NPC1 protein was detected with a polyclonal NPC1 rabbit antibody raised against a peptide corresponding to a 19-amino acid residues (1256–1274) peptide in the C-terminus of human NPC1, used at 1:500 dilution. Final detection was done by chemiluminescence using the ECL detection kit (Amersham Pharmacia Biotech).

## Results

### Spectrum of NPC1 Mutations

Analysis of cDNA and genomic DNA from 30 patients belonging to the NPC1 complementation group led to the identification of 32 different mutations, 26 of which were novel. A compendium of the mutant alleles is presented in table 3, and their location on a model of the NPC1 protein is represented schematically in figure 1. The largest number (25) of mutant alleles contained point mutations that led to single-amino acid substitutions. None of the novel substitutions was found in the control population of 95 individuals tested. Other abnormalities included two nonsense mutations, two 1-bp deletions, one 381-bp insertion, one 14-bp duplication, and one splice mutation. Mutations were widely scattered and affected all domains of the protein except for the NPC1 domain in the N-terminal end. Three of the five frameshift mutations were in the N-terminal luminal loop, leading to complete or near-complete truncation of the protein, and three missense mutations were located in the putative SSD. Among them, the Q775P allele was found to be homozygous in one family (5) and heterozygous in another (20). It was noteworthy that 11 (i.e., one-third) of the 32 identified mutations were located on the luminal cysteine-rich loop between transmembrane domains 8 and 9. Except for a nonsense mutation, these mutations all resulted in a single-amino acid substitution. Two different changes of the same nucleotide, G2974C (24) and G2974A (27) were observed, both resulting in the G992R substitution. Because of our particular selection of families, the I1061T allele, documented elsewhere as the most common NPC1 mutation (Millat et al. 1999), constituted only 7 of the 60 alleles, but P1007A (Greer et al. 1999) appeared as the second-most-recurrent allele (4/60) in this study.

**Table 3**

**Mutations in 30 Unrelated Patients with NPC1**

PATIENT IDENTIFICATION (PHENOTYPE)	LOCATION	NUCLEOTIDE CHANGE <sup>a</sup>	EFFECT ON PROTEIN	
			Amino Acid Change	Affected Domain <sup>b</sup>
1 98119 (Cl)	Exon 2	72delC 72delC or deletion	Frameshift from codon 24 <sup>c</sup> PTC+33 aa	Stop at the end of signal peptide
2 80001 (Cl)	Exon 6	763dupl 14 bp <sup>c</sup> (GCCCCAGCCCCAC)	Frameshift from codon 255 <sup>c</sup> PTC+59 aa	Stop before TM1
3 87024 (Cl)	Exon 6	845delT <sup>c</sup>	Frameshift from codon 282 <sup>c</sup> PTC+27 aa	Stop within TM 1
4 91029 (Cl)	Exon 19	C2819A <sup>c</sup>	S940X <sup>c</sup>	Stop within the cysteine-rich luminal loop between TM 8 and 9
5 81057 (Cl)	Exon 15	A2324C <sup>c</sup>	Q775P <sup>c</sup>	Sterol-sensing domain– TM7; residue conserved in SCAP
6 83024 (Cl)	Exon 21	G3160A <sup>c</sup>	A1054T <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
7 83049 (Cl)	Exon 6	C709T <sup>d</sup>	P237S	N-terminal loop
8 82052 (Cl)	Exon 8	G1211A	R404Q	Luminal, between TM 2 and 3
	Exon 12	T1892G	M631R	TM3
9 79011 (Cl)	?	?	?	?
	Exon 9	G1553A <sup>d</sup>	R518Q+splicing mutation	Between TM 2 and 3
10 96115 (Cl)	?	?	?	?
	Exon 14	T2171C	L724P	Sterol-sensing domain
11 82042 (Cl)	Intron 24	IVS24+1G→C	Frameshift after codon 1197 PTC+3 aa	Exon 24 skipping+frameshift
	Exon 23	G3557A <sup>c</sup>	R1186H	Between TM 11 and 12
12 95085 (Cl)	?	?	?	?
	Exon 23	G3503A <sup>c</sup>	C1168Y <sup>c</sup>	Between TM 11 and 12 (cytosolic loop)
13 90104 (Va)	Exon 20	C3019G	P1007A	Cysteine-rich luminal loop between TM 8 and 9
	Exon 22	G3428A	W1143X	Between TM 11 and 12
14 93094 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 6	G724C	D242H	N-terminal loop

15 91048 (Cl)	Exon 21	T3182C <sup>c,f</sup>	I1061T <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
16 98009 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 12	C1814T	A605V	Loop between TM 2 and 3
17 90096 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G <sup>g</sup>	P1007A	Cysteine-rich luminal loop between TM 8 and 9
18 94139 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G <sup>g</sup>	P1007A	Cysteine-rich luminal loop between TM 8 and 9
19 92114 (Va)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 20	C3019G <sup>g</sup>	P1007A	Cysteine-rich luminal loop between TM 8 and 9
20 95070 (Va)	Exon 20	G2956A	G986S	Cysteine-rich luminal loop between TM 8 and 9
	Exon 15	A2324C	Q775P	Sterol-sensing domain – TM7
21 97089 (Va)	Exon 19	C2829G <sup>c</sup>	I943M <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
22 90002 (Va)	Exon 19	G2848A <sup>c</sup>	V950M <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
23 96157 (Cl)	Exon 16	A2474G	Y825C	Cytosolic loop between TM 7 and 8
	?	?	?	?
24 96157 (Va)	Exon 20	G2974C <sup>c</sup>	G992R <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
25 97014 (Cl)	Exon 21	T3182C	I1061T	Cysteine-rich luminal loop between TM 8 and 9
	Exon 8	T1133C	V378A	Loop between TM 2 and TM3
26 96084 (Cl)	Exon 18	A2621T <sup>c</sup>	D874V <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
27 91144 (Va)	Exon 20	G2974A	G992R	Cysteine-rich luminal loop between TM 8 and 9
	Junction exon 1/exon 2	57ins381bp	Frameshift from codon 19 PTC+19 aa	Stop within signal peptide
28 86065 (Cl)	Exon 19	G2801A <sup>c,g</sup>	R934Q <sup>c</sup>	Cysteine-rich luminal loop between TM 8 and 9
29 97114 (Cl)	Exon 6	T815G <sup>c</sup>	M272R <sup>c</sup>	TM1
30 97119 (Cl)	Exon 19	G2830A	D944N	Cysteine-rich luminal loop between TM 8 and 9
	Exon 22	T3425C	M1142T	TM 10

NOTE.—PTC = premature stop codon. TM = transmembrane domain. Cl = classic biochemical phenotype. Va = variant biochemical phenotype.

<sup>a</sup> Counted from the adenosine residue of the initiation codon.

<sup>b</sup> The putative spanning membrane domains of the NPC1 protein were determined according to Davies and Ioannou (2000).

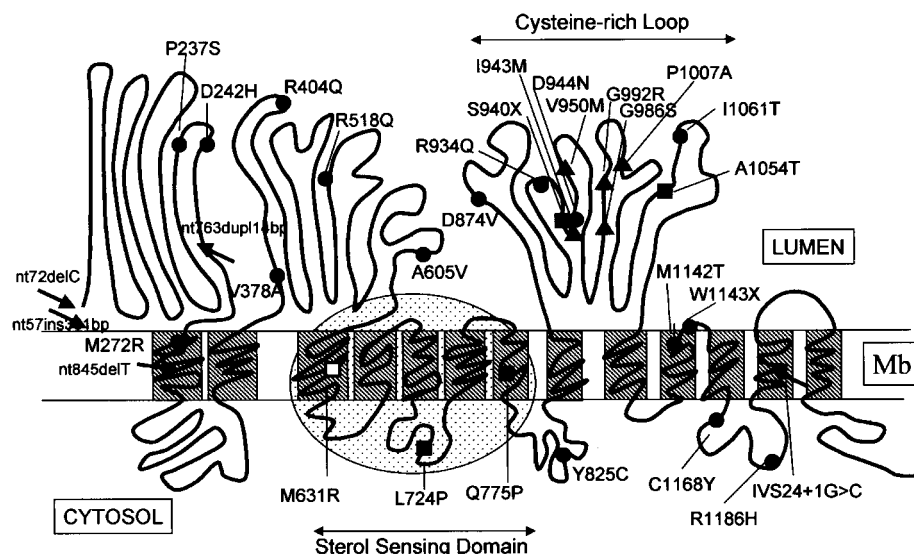
<sup>c</sup> Mutation was homozygous in the patient.

<sup>d</sup> Yamamoto et al. 1999.

<sup>e</sup> Carstea et al. 1997.

<sup>f</sup> Millat et al. 1999.

<sup>g</sup> Greer et al. 1999.



**Figure 1** Topology of the mutations identified in 30 patients on an NPC1 protein model and genotype/phenotype correlations. The schematic NPC1 protein model is drawn as proposed by Davis and Ioannou (2000). The hatched areas indicate the putative transmembrane domains. The dotted oval frame delimits the SSD. →, Frameshift mutations, all of them observed in patients with a severe infantile neurological form. ■, Missense or nonsense mutation definitively associated to a severe infantile neurological form. □, missense mutation likely correlated with a severe infantile neurological form. ▲, Missense mutation definitively correlated with a variant biochemical phenotype. ●, Other missense mutation.

#### *Correlations between Genotypes and the Classic or Variant Biochemical Phenotypes*

One aim of our study was to pinpoint possible differences between mutations associated with the classic or the variant biochemical phenotypes (severe or mild impairment of cellular cholesterol trafficking, respectively) on the basis of the observation that the biochemical phenotype was constant within a sibship (Vanier et al. 1991a). In the homozygous state, all frameshift, splice or nonsense mutations and a number of missense mutations (M272R, Q775P, D874V, R934Q, A1054T, I1061T, and C1168Y) led to the classic biochemical phenotype (fig. 1 and table 3). This was also the case for several combinations of two missense mutations (patients 7, 14, 16, 25, and 30) or of L724P associated with a frameshift mutation (patient 10) (table 3).

Among the nine patients with the less common variant phenotype, four were found to carry one P1007A allele, combined either with I1061T (patients 17, 18, and 19) or to a nonsense mutation, W1143X (patient 13). I1061T has definitively been correlated with a classic phenotype when in the homozygous state (Millat et al. 1999), and one P1007A allele thus appeared sufficient to maintain some degree of cholesterol trafficking. These data suggested that the P1007A mutation was a common mutant allele associated with the variant biochemical phenotype.

This hypothesis was confirmed by investigation of genomic DNA in a total population of 55 patients with

NPC1, using a simple PCR test based on introduction of an *NheI* restriction site. The P1007A allele was never observed in the 22 patients with a classic biochemical phenotype but constituted 15/66 alleles (22.7%) in the 33 patients studied who had the variant phenotype (9 from the present mutational study and 24 additional ones).

Four additional mutant alleles could clearly be ascribed to a variant phenotype: I943M (patient 21), V950M (patient 22) and G992R (patient 24) were all found in the homozygous state, and G986S (patient 20) was found in combination with Q775P which is clearly associated with a classic phenotype (patient 5). An intriguing observation is that these four mutations, as well as P1007A, are clustered within a small region of the cysteine-rich luminal loop between transmembrane domains 8 and 9 (fig. 1).

#### *Correlations between Genotypes and Mutant NPC1 Protein Studied by Western Blot*

Selected cell lines with missense mutations, most of them homozygous or associated with a frameshift mutation, were studied by immunoblotting (fig. 2). The homozygous 845delT cell line, which gives rise to very early premature termination of the protein, was tested to establish the specificity of the affinity-purified polyclonal antibody, and, as expected, no detectable band was seen in the region of interest. Normal fibroblasts gave a double band of ~170 and ~190 kD. Essentially no detectable



protein was repeatedly found in three cell lines with missense mutations: homozygous Q775P and L724P, both located in the SSD, but also A1054T in the cysteine-rich region. Furthermore, a clearly diminished amount of the protein was found in cells with the C1168Y mutation. In all other mutations studied, including the classic I1061T, a substantial amount of NPC1 protein could be detected. Definite conclusions about an apparent small reduction of protein in some mutations are difficult to draw, since repeated experiments on different cell pellets could show a variation of at least twofold in levels of expression of the protein, in both normal cells and mutant cells. We observed that the protein was unstable in dilute solution, but variations under strict experimental conditions suggest the influence of yet-undefined factors that regulate expression of NPC1. Interestingly, NPC2 cells carrying the mutation E20X on the *HE1* gene (fig. 2) or another mutation (Yamamoto et al. 2000) invariably showed NPC1 protein levels well above those observed in the control cells.

#### Correlations with the Clinical Phenotype

The three patients with a frameshift mutation and premature stop codon (patients 1, 2, and 3) or a nonsense mutation (patient 4) on both alleles all had an early neurological onset and a short life span. This was also the case for all patients with missense mutations for which immunoblotting revealed a lack of NPC1 protein. Patients 5 and 6 carried a homozygous missense mutation in the SSD or the cysteine-rich luminal loop, respectively. Patient 10, who had a SSD missense mutation associated with a splice mutation leading to exon-24 skipping, had a slightly later neurological onset, but the

disease is progressing very rapidly. For patient 8, one allele carried a mutation in the SSD of an amino acid conserved in the *patched* gene, but the second allele remained unidentified. The significantly reduced amount of NPC1 protein observed in fibroblasts of patient 12, who had the C1168Y mutation, also correlated with the relatively severe late-infantile neurological phenotype.

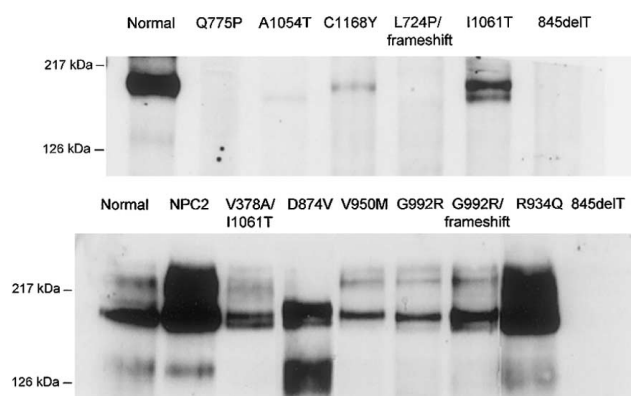
Conversely, the finding of a near-normal amount of NPC1 protein was of limited predictive value. This pattern was shared by patients with a neurological juvenile onset (patient 15, I1061T), a neurological adult form (patient 22, V950M), or a nonneuronopathic adult form (patient 24, G992R, and patient 25, I1061T/V378A). On the other hand, correlations could clearly be drawn concerning the clinical expression of some missense mutations or combination of missense mutations. The combination of the two most common alleles, I1061T/P1007A, was encountered in three families (17, 18, and 19; five patients) and resulted in a juvenile onset of symptoms and a significantly slower progression of the disease than in homozygous I1061T patients. P1007A combined with a nonsense mutation (patient 13) resulted in a late-infantile neurological form. Homozygous V950M corresponded to an adult onset of neurological symptoms. Study of two adults with splenomegaly and no neurological symptoms (patients 24 and 25) led to the conclusion that G992R and V378A were responsible for this atypical mild phenotype. Interestingly, G992R had only a mild effect on cholesterol trafficking, whereas V378A was associated with severe biochemical alterations, demonstrating that the block in cholesterol transport does not correlate with clinical severity, as discussed elsewhere (Vanier et al. 1991b; Vanier and Suzuki 1998). Future studies should disclose whether correlations would be tighter with the newly discovered, possibly more relevant permease function of the NPC1 protein (Davies et al. 2000).

Patients 26 and 27 are still too young for conclusions to be drawn about their potential neurological subtyping. Mutations found in patients 28–30, initially studied to facilitate prenatal diagnosis, are reported because M272R and M1142T (which affects an amino acid conserved in *patched*) were the only two missense mutations found in our study in transmembrane domains outside of the SSD. R934Q was reported elsewhere in association with I1061T (Greer et al. 1999). But since the first affected child in those families died early, of liver failure, the complete phenotype associated with these mutations remains unknown.

#### Discussion

##### General Distribution of NPC1 Mutations

Although the number of known NPC1 mutations appears to be ~100, taking into account data presented by



**Figure 2** Western blot of NPC1 protein in cultured fibroblasts from control subjects, 12 patients with NPC1, and 1 patient with NPC2. A C-terminal affinity-purified polyclonal antibody was used. Cells from a patient with a 845delT frameshift mutation served as a negative control. Eight patients with NPC1 had a homozygous missense mutation (lanes with a single label), and three were compound heterozygotes (lanes with a dual label). The NPC2 cell line carried a homozygous E20X mutation of the *HE1* gene.

seven groups at international meetings (Patterson et al. 2001), few studies have been published except in abstract form. In a survey of 14 Japanese patients with NPC1, with a majority of late-infantile cases, the 14 missense mutations and one in-frame deletion were widely distributed on NPC1 cDNA (Yamamoto et al. 1999, 2000). This was at variance with the initial data on nine patients reported by Carstea et al. (1997), where most mutations were found to affect the carboxy-terminal third of the protein. A later survey of 13 families originating from Canada and Maryland, restricted to the last six NPC1 exons and boundaries, indeed allowed identification of 20/26 mutant alleles (Greer et al. 1999). Greer and colleagues further observed that six of the eight missense mutations found in these patients—including G992W, which is characteristic of Nova Scotian patients—were clustered in a conserved NPC1-specific cysteine-rich loop. More recent topological analysis of the NPC1 protein by Davies and Ioannou (2000) indicated that the most common missense mutation, I1061T (Greer et al. 1999; Millat et al. 1999), initially thought to affect a transmembrane domain, also located to this luminal loop. In the present study, which included mostly white patients but covered a wider clinical spectrum, nearly all domains of the NPC1 protein—except for the leucine-zipper region—were affected. Eleven of the twenty-five identified missense mutations and four frameshift mutations were located in the first two-thirds of the protein, suggesting that the discrepancy between previous results in Japanese versus North American populations might be explained, in part, by differences in phenotypes of the patients. Mutagenesis studies indicated that both the leucine-zipper motif and the putative SSD were particularly critical regions (Watari et al. 1999). Mutations in the SSD have been identified by Yamamoto et al. (2000) (F703S) and in this report (M631R, L724P, and Q775P), but no naturally occurring mutations have been reported so far that affect the NPC1 leucine-zipper domain. In the present study, 14 of the 30 patients showed a proven or almost certain homozygous mutation, which greatly facilitated establishment of genotype/phenotype correlations.

#### *Perinatal Liver Disease Independent of the NPC1 Mutation*

It is well known that the neonatal, severe cholestatic form of NPC and neurological forms of the disease may occur in the same sibship (Vanier et al. 1991a); this is further illustrated in this study by families 2, 3, and 5. Most of the published families have shown an association with an infantile neurological form (Patterson et al. 2001), but one of the sibs in the family described by Jaeken et al. (1980) had a neurological juvenile-onset form. We recently found one patient with fetal hydrops/

ascites to be homozygous for the I1061T mutation (M. T. Vanier and G. Millat, unpublished data). His affected elder brother, now 24 years old, presented with the expected neurological juvenile form (Millat et al. 1999). Thus, fatal liver disease in a first affected child (patients 28–30) gives no clue as to the possible neurological course in other sibs, unless the mutations correspond to a known neurological subtype. Remarkably, the rapidly fatal neonatal hepatic forms we studied so far all showed severe alterations of cellular cholesterol trafficking (M. T. Vanier, unpublished data).

#### *Missense Mutations in SSD as well as Frameshift and Nonsense Mutations Lead to Absence of Stable NPC1 Protein and Severe Neurological Phenotype*

In patient 1, initially described as having “lactosylceramidosis” (Dawson et al. 1971) and later reclassified as having NPC (Wenger et al. 1975; Vanier et al. 1988), a frameshift occurs at the end of the putative signal peptide. Patients in families 2 and 3 (Vanier et al. 1988; Kanoun et al. 1989) also have a very early NPC1 termination caused by a frameshift mutation, whereas those in family 4 have a nonsense mutation. The very severe neurological and biochemical phenotype observed in these families thus is well in line with the nature of the molecular lesion. The Q775P missense molecular lesion found for family 5 is of particular interest, both as the first naturally occurring homozygous mutation described in the NPC1 SSD (amino acids 615–797) and because it affects a SCAP-conserved residue. NPC1 protein (fig. 2) was undetectable both in fibroblasts of this patient and in those of patient 10, in whom another SSD-located substitution, L724P, was associated with a truncation of the C-terminus that should result in abnormal targeting of the protein (Watari et al. 1999). In patient 8, who had a M631R alteration, the second allele remained unidentified, but this patient also had a very severe course of the disease. Absence of detectable levels of NPC1 protein was reported by Yamamoto et al. (2000) in a Japanese patient with a F703S/S813X combination. Our results are in agreement with those of the latter authors, who were the first to point out a correlation between absence or near absence of NPC1 protein and the most severe clinical presentations (Yamamoto et al. 2000). Conversely, SSD mutants created by site-directed mutagenesis (Y643S, Y643C, and P691S), studied after transfection in CT-60 CHO cells, showed the presence (although in reduced amount) of NPC1 protein (Watari et al. 1999). Since we obtained similar results after transfection of Q775P cDNA to mutant FR4 CHO cells (K. Chikh, G. Millat, C. Tomasetto, K. Ohno, and M. T. Vanier, unpublished data), a likely explanation would be that overexpression masks the instability of the native protein. Brain glycolipids, studied in patients

1 (Dawson et al. 1972) and 8 (Vanier 1999), showed striking abnormalities. Taken together, these data clearly show that mutations affecting the putative SSD of the NPC1 protein are particularly deleterious, since they invariably correspond to the most severe neurological form of the disease.

Most missense alterations located to other regions of the NPC1 protein also severely affect cholesterol processing. A majority have a lesser impact on the stability of the protein as seen from immunoblotting, and a varying effect on the clinical picture, as illustrated by C1168Y (late-infantile neurological onset, patient 12), I1061T (juvenile neurological onset; Millat et al. 1999), and V378A, which apparently underlines the nonneurological adult phenotype observed in patient 25.

In spite of the fact that recent data show that NPC1 has an unexpected transmembrane molecular-pump activity and suggest that it is a permease that can transport certain lipids, but apparently not cholesterol, across membranes (Davies et al. 2000), studies in patients definitively highlight a key role of the SSD for the *in vivo* function of the protein.

#### *Cysteine-Rich Luminal Loop Appears as a Functionally Complex Domain*

Greer et al. (1999) were the first to highlight the functional significance of the cysteine-rich domain on the basis of mutational studies in patients. This domain has resemblance to the RING-finger motif regulatory domain of protein kinase C. Watari et al. (2000) further showed that this domain can bind zinc. From the studies of Greer et al. (1999), Yamamoto et al. (2000), and ourselves (Millat et al. 1999 and the present work), no fewer than 16 different missense mutations have already been ascribed to the cysteine-rich domain, which spans amino acids 855 through 1098 (Davies and Ioannou 2000). The first mutation identified, G992W, characterizes patients from Nova Scotia (former Niemann-Pick type D), with a juvenile onset and slowly progressing neurological disease (Greer et al. 1998). Despite the fact that Nova Scotian patients are of Acadian extraction, G992W has not been found so far in French patients. We identified two other base alterations on the same codon, both leading to the G992R substitution. Patient 24, who has isolated splenomegaly (Frölich et al. 1990) and is homozygous for this mutation, still has no neurological signs of NPC at age 66 years (K. Harzer, unpublished data). Cultured fibroblasts from this patient; from patient 27, who has G992R and a frameshift mutation on the other allele; and from patients from Nova Scotia (Byers et al. 1989) all behaved as biochemical “variants,” leading to the conclusion that codon 992 is not essential for cholesterol transport. On the other hand, the most recurrent I1061T mutation, initially de-

scribed by us (Millat et al. 1999) and by Greer et al. (1999), which constitutes 15%–18% of mutated alleles in countries like the United Kingdom, France, or the United States, is correlated with severe alterations of cholesterol trafficking (the classic phenotype) and a juvenile-onset neurological disease. Immunoblotting studies revealed a quite good level of NPC1 protein in cells homozygous for both the G992R and I1061T mutations. Conversely, the present study has identified the A1054T mutation, for which no NPC1 protein was detectable, and which correlated with a severe infantile neurological onset form (patient 6). Thus, at variance with what was seen for the SSD, consequences of naturally occurring missense mutations affecting the cysteine-rich luminal loop appeared highly variable.

#### *P1007A is a Common Mutated Allele Associated with the Variant Biochemical Phenotype*

One patient homozygous for the P1007A mutation, categorized as having the biochemically variant phenotype, was described by Greer et al. (1999). We found four additional patients with the variant phenotype in whom this allele was combined with I1061T or a nonsense mutation and further showed a high prevalence of this allele, 22.7%, in a total population of 33 patients with the variant phenotype. Systematic screening for this mutation thus appears justified in patients with the variant phenotype.

#### *Seven Variant Mutant Alleles So Far Identified Locate to the Cysteine-Rich Luminal Loop*

From the study of patients with the P1007A and G992R mutations, we concluded that a single variant allele apparently leads to a variant phenotype, irrespective of the nature of the second allele. Apart from G992W, G992R, and P1007A discussed above, I943M, V950M, G986S (identified in this report) and V889M (Yano et al. 1996; Yamamoto et al. 2000) could all be classified as variant alleles. Although a recent study in Portuguese patients indicated that some variant mutations may affect another domain (Ribeiro et al., *in press*), those reported so far all located to the cysteine-rich loop, and most of them were clustered within a small region (codons 943–1007) of this loop. The present observation suggests that part of the cysteine-rich domain could be associated with a NPC1 function not tightly associated with cholesterol trafficking. The NPC1 protein seems to be implicated in glycolipid transport (Blanchette-Mackie 2000; Zhang et al. 2001), and, since glycolipid abnormalities have been found in brain of patients with the variant phenotype (Martin et al. 1984; Vanier 1999), more-detailed studies are indicated. Future studies on the three-dimensional structure of the cysteine-luminal loop might also provide new insights on the functional

complexity of this domain. Above all, how variant mutations specifically relate to the molecular-pump activity of NPC1 recently described by the group of Ioannou (Davies et al. 2000) is a particularly intriguing question.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, <http://www.ncbi.nlm.nih.gov/Genbank/index.html> (for NPC1 cDNA [accession number AF002020])  
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for Niemann-Pick C [MIM 257220])

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## Niemann-Pick Disease Type C: Spectrum of *HE1* Mutations and Genotype/Phenotype Correlations in the NPC2 Group

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In Niemann-Pick disease type C (NPC), a genetic heterogeneity with two complementation groups—NPC1, comprising >95% of the families, and NPC2—has been demonstrated. Mutations in the *NPC1* gene have now been well characterized. *HE1* was recently identified as the gene underlying the very rare NPC2. Here we report the first comprehensive study of eight unrelated families with NPC2, originating from France, Algeria, Italy, Germany, the Czech Republic, and Turkey. These cases represent essentially all patients with NPC2 who have been reported in the literature, as well as those known to us. All 16 mutant alleles were identified, but only five different mutations, all with a severe impact on the protein, were found; these five mutations were as follows: two nonsense mutations (E20X and E118X), a 1-bp deletion (27delG), a splice mutation (IVS2+5G→A), and a missense mutation (S67P) resulting in reduced amounts of abnormal *HE1* protein. E20X, with an overall allele frequency of 56%, was established as the common mutant allele. Prenatal diagnosis was achieved by mutation analysis of an uncultured chorionic-villus sample. All mutations except 27delG were observed in a homozygous state, allowing genotype/phenotype correlations. In seven families (with E20X, E118X, S67P, and E20X/27delG mutations), patients suffered a severe and rapid disease course, with age at death being 6 mo–4 years. A remarkable feature was the pronounced lung involvement, leading, in six patients, to early death caused by respiratory failure. Two patients also developed a severe neurological disease with onset during infancy. Conversely, the splice mutation corresponded to a very different clinical presentation, with juvenile onset of neurological symptoms and prolonged survival. This mutation generated multiple transcripts, including a minute proportion of normally spliced RNA, which may explain the milder phenotype.

### Introduction

Niemann-Pick disease type C (NPC [MIM 257220 and MIM 601015]) is an autosomal recessive lysosomal lipid-storage disorder with a protean clinical presentation (Patterson et al. 2001). The disease is most commonly characterized by hepatosplenomegaly and a severe progressive neurological dysfunction with varying age at onset and varying later course. The cellular hallmark of NPC is a late-endosomal/lysosomal accumulation of endocytosed unesterified cholesterol. In tissues, there is a complex pattern of accumulating lipids that differs between nonneural

organs and brain, with no overall increase of sphingomyelin and cholesterol in the latter (Vanier and Suzuki 1996; Blanchette-Mackie 2000; Patterson et al. 2001).

A genetic heterogeneity was established in NPC by somatic-cell hybridization and linkage analysis, defining two different genetic complementation groups, NPC1 and NPC2 (Steinberg et al. 1994; Vanier et al. 1996). Because identical cellular and biochemical phenotypes were observed in patients belonging to the two genetic complementation groups (Vanier et al. 1996; Christomanou et al. 2000), it has been concluded that both gene products may function either in tandem or sequentially (Vanier et al. 1996). The *NPC1* gene (mapped to 18q11), mutated in 95% of families with NPC, was isolated by positional cloning and has been fully characterized (Carstea et al. 1997; Morris et al. 1999). The 4.7-kb *NPC1* cDNA sequence encodes a 1,278-amino-acid protein, shown to contain 13 transmembrane domains, three large luminal loops, four small luminal loops, six small cytoplasmic loops, and one cytoplasmic tail (Davies and Ioannou 2000). It also contains a sterol-

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**Table 1****Clinical and Biochemical Survey of Families with NPC2**

Patient ID (Ethnicity)	Age at Death	Clinical Summary	Consanguinity?	LDL-Induced Cholesteryl-Ester Formation <sup>a</sup>	Reference(s)
Case 1 (French)	6 mo	Female; hydramnios, cesarean section at 32 wk gestation, respiratory distress from birth, moderate neonatal cholestatic icterus at age 4–30 d, hepatosplenomegaly, respiratory failure (two affected siblings were diagnosed prenatally, and the pregnancies were terminated)	No	<10	
Case 2 (French)	6 mo	Female; fetal ascites (that recessed) at 23 wk gestation, hepatosplenomegaly at birth, cholestatic icterus at age 1 mo, severe respiratory insufficiency from age 3 mo, respiratory failure	No	50	Pin et al. (1990), Vanier et al. (1996) (case 16)
Case 3 (Italian)	6 mo	Female; neonatal cholestatic icterus, hepatosplenomegaly, respiratory failure (three further affected siblings were diagnosed prenatally, and the pregnancies were terminated)	No	75	Steinberg et al. (1994), Vanier et al. (1996) (case 19), Nau-reckiene et al. (2000)
Case 4 (German)	7 mo	Female; neonatal period uneventful, severe interstitial pneumonia/hepatosplenomegaly/respiratory insufficiency/failure at age 3 mo (a brother with “neonatal hepatitis” and severe lung involvement had died at age 6 wk)	Yes	320	Schofer et al. (1998), Vanier et al. (1996) (case 28)
Case 5 (Algerian)	19 mo	Male; neonatal cholestatic icterus until age 4 mo, then pulmonary involvement; severe motor development delay from age 7–9 mo (able to sit only at age 15 mo, no further progress); growth retardation, increasing pulmonary fibrosis, oxygen dependency, respiratory failure	Yes	10	Vanier et al. (1996) (case 2)
Case 6 (Czech)	4 years	Female; failure to thrive (with poor feeding), hepatosplenomegaly and mild delay of motor milestones at age 1 year, severe lung involvement, oxygen dependent from age 20 mo, quadroparesis	No	45	Christomanou et al. (2000), Elleder et al. (2001)
Case 7 (Turkish)	Alive (at 20 mo)	Female; no neonatal cholestatic icterus; respiratory distress and pulmonary infiltration at age 11 mo, (hepato-)splenomegaly; new acute respiratory problems at age 13 mo, at which psychomotor development was normal; hypotonia and gait problems from age 18 mo (an elder brother had splenomegaly and died, of respiratory distress, at age 6 mo in Turkey)	Yes	10	
Case 8 (Algerian)	Alive (at 21 years)	Female; splenomegaly at age 6.5 years, school problems, epileptic fits at age 11 years, mental retardation, slow progression, still able to walk normally at age 21 years (an affected sister with quite similar course was still alive at age 27 years)	Yes	380	Vanier et al. (1996) (case 27)

<sup>a</sup> After 4.5 h, expressed as pmol/mg cell protein, studied as described by Vanier et al. (1991) (normal values  $2,950 \pm 1,200$ ).

sensing domain with the same orientation as that of HMG-CoA reductase and SCAP (sterol regulatory element binding protein [SREBP] cleavage activation protein) (Davies and Ioannou 2000). Approximately 100 different NPC1 mutations have already been identified in patients with NPC1 (Carstea et al. 1997; Greer et al. 1998, 1999; Yamamoto et al. 1999, 2000; Millat et al. 2001; Ribeiro et al. 2001; Sun et al. 2001); they have been shown to affect all domains of the protein—with some predilection, however, for a conserved cysteine-rich luminal loop (Greer et al. 1999; Millat et al. 2001). Only three of these mutations—I1061T, G992W, and P1007A—appear to occur quite frequently (Greer et al. 1998; Millat et al. 1999, 2001). The NPC1 protein resides in late endosomes and interacts transiently with lysosomes and with the *trans*-Golgi network (Higgins et al. 1999; Neufeld et al. 1999). The exact cellular function of NPC1 and the role that it has in intracellular trafficking of lipids remain unclear (Liscum and Munn 1999; Blanchette-Mackie 2000; Cruz et al. 2000; Davies et al. 2000; Ory 2000). A number of studies suggest that NPC1 has a key role in the modulation of vesicular trafficking of cholesterol and of glycolipids (Neufeld et al. 1999; Blanchette-Mackie 2000; Zhang et al. 2001), but recent data suggest that NPC1 is a permease acting as a transmembrane efflux pump (Davies et al. 2000).

To date, only eight families belonging to the minor NPC2 complementation group (MIM 601015), estimated to account for 5% of families with NPC, have been documented (Steinberg et al. 1994, 1996; Vanier et al. 1996; Christomanou et al. 2000; Naureckiene et al. 2000; Elleder et al. 2001). Recently, by a proteomic approach, the HE1 gene (mapped to 14q24) was identified as the disease-causing gene in patients with NPC2 (Naureckiene et al. 2000). For practical reasons and by analogy with NPC1, we propose henceforth to use the name “HE1/NPC2.” HE1/NPC2 is ~13.5 kb long and is composed of five exons of size 78–342 bp (Naureckiene et al. 2000). Analysis of the 822-bp cDNA sequence showed an open reading frame of 453 bp, followed by a 3' UTR of 327 bp comprising most of exon 5 (Kirchhoff et al. 1996). Translation of HE1/NPC2 leads to a mature protein of 132 amino acid residues, with a theoretical molecular mass of 14.5 kD.

The HE1/NPC2 glycoprotein (GenBank) was first characterized as a major secretory protein in the human epididymis (Kirchhoff et al. 1996) and was also shown to bind cholesterol (Baker et al. 1993; Okamura et al. 1999). It is now known to be a small, soluble, ubiquitously expressed lysosomal protein, the functional alteration of which leads to an NPC phenotype (Naureckiene et al. 2000). In the original study establishing HE1 as the gene underlying NPC2, severe mutations leading to absence of HE1 protein were reported in two patients (Naureckiene et al. 2000). One patient carried

**Table 2****Oligonucleotide Sequences**

Oligonucleotide	Sequence <sup>a</sup> (5'→3')
NPC2-ex1s	gctgtgggtactgtgacagg
NPC2-ex1as	tccaaggctcagcctggccg
NPC2-ex2s	ctaaatgggagagcagacac
NPC2-ex2as	ccctccattcccatgcttattc
NPC2-ex3s	atgcttgactgtgtagtac
NPC2-ex3as	tgatgcctaacaccgcaccta
NPC2-ex4s	ggagttagggaagttgtactg
NPC2-ex4as	tggcactgatttagtttcagtc
NPC2-ex5s	gcctgcacagtggaggaaag
NPC2-ex5as	ggcctgccttccatggttct
NPC2-ADNcs(3) <sup>b</sup>	<sup>-21</sup> CTTGGAACCTTCGTTATCCGCG <sup>-1</sup>
NPC2-ADNcas(2) <sup>b</sup>	<sup>535</sup> TGTTGAAGCAGCAGAGCTGG <sup>516</sup>

<sup>a</sup> Exonic sequences are given in uppercase, and intronic sequences are given in lowercase.

<sup>b</sup> Counted from the adenosine residue of the initiation codon.

a homozygous E20X mutant allele, and the other patient was a compound heterozygote for the E20X mutation and for a single-nucleotide deletion (311delA) in exon 2. In the present report, the molecular defect has been characterized in a further seven unrelated families with NPC2 and has been applied to rapid first-trimester prenatal diagnosis of the condition. Furthermore, the clinical and biochemical phenotypes in the eight families can now be better understood in terms of the underlying genetic mutations.

## Subjects, Material, and Methods

### Subjects and Biological Material

Fibroblast strains from eight unrelated patients with NPC2 (six previously documented cases and two from newly diagnosed families) were used in the study. The diagnosis of NPC disease had been assessed by filipin staining and by measurement of LDL-induced cholesteryl-ester formation, as described elsewhere (Vanier et al. 1991) (table 1). Complementation analysis by somatic-cell hybridization and by filipin staining had previously been documented in a majority of the cases (Steinberg et al. 1994; Vanier et al. 1996; Schofer et al. 1998) and, in the remaining three families (cases 1, 6, and 7), was performed by the simplified procedure described by Schofer et al. (1998). Genomic DNA was also available from parents of cases 1, 3, and 6–8 and from four affected fetuses who would have been siblings of cases 1 and 3. The main clinical and biochemical features of the patients with NPC2 are summarized in table 1.

### Genomic DNA Extraction

Genomic DNA was extracted from cultured skin fibroblasts, peripheral blood leukocytes, and uncultured

**Table 3****Spectrum of Mutations Identified in Patients with NPC2**

Patient (Cell Line)	Genotype	Nucleotide Change(s) <sup>a</sup>	Location(s)	Predicted Protein Alteration(s)
Case 1 (20082)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 2 (88082)	E20X/27delG	G58T/27delG	Exon 1/exon 1	Stop at codon 20/frameshift from codon 9; premature termination codon + 24 amino acids
Case 3 (88057/NIH99.04 <sup>b</sup> )	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 4 (93104)	E118X/E118X	G352T	Exon 3	Stop at codon 118
Case 5 (89095)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 6 (96059)	E20X/E20X	G58T	Exon 1	Stop at codon 20
Case 7 (21033)	S67P/S67P	T199C	Exon 3	S67P
Case 8 (82017/NIH94.85 <sup>b</sup> )	IVS2+5G→A/IVS2+5G→A	...	Intron 2	Multiple mRNAs (see text and fig. 1)

<sup>a</sup> Counted from the adenosine residue of the initiation codon.

<sup>b</sup> New code, assigned after the strains were transferred to the National Institutes of Health (NIH) within the frame of a collaboration between either A.H.F. or M.T.V. and Dr. P. G. Pentchev.

chorionic-villus samples, by standard procedures (Jeanpierre 1987; Miller et al. 1998)

### Sequencing

After amplification, PCR products were purified and directly sequenced in both directions by the Thermo-sequenase cycle sequencing kit and were primer labeled with  $\gamma$ [<sup>33</sup>P]-ATP at 3,000 Ci/mmol (Amersham Pharmacia Biotech).

### RNA Isolation and cDNA Synthesis

Total RNA was isolated from fibroblast monolayers by Trizol reagent (Life Technologies), and reverse transcription was performed with oligo-dT primer, by the First Strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

### Subcloning

cDNA from case 8 was amplified by primers NPC2-ADNcs(3) and NPC2-ADNcas(2) (table 2). PCR products were purified and subcloned into pGEM-T vector (Promega). After transformation, 32 isolated subclones were sequenced in both directions.

### Mutation Detection of the E20X Nonsense Mutation in Genomic DNA

Exon 1 of *HE1/NPC2* was PCR amplified by the primers NPC2-ex1s and NPC2-ex1as (table 2). The 40 PCR cycles each included steps of 1 min at 92°C, 1 min at 62°C, and 1 min at 72°C. Because the mutation created a *StuI* restriction site, the 325-bp PCR product was digested for 2 h at 37°C by *StuI* and was analyzed on 3% Nusieve-GTG gels (BioWhitaker Molecular Applications).

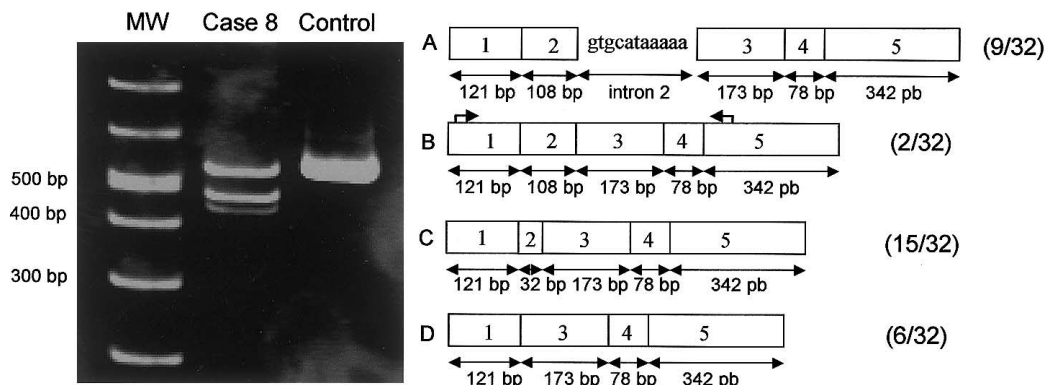
### Western Blot Studies

For western blot studies, fibroblast lysates were prepared as described by Naureckiene et al. (2000), and soluble protein (10  $\mu$ g protein/lane) was separated on 12% SDS-acrylamide gels. The blot was probed with rabbit antibodies to HE1/NPC2 (Naureckiene et al. 2000), followed by chemiluminescence by the ECL detection kit (Amersham Pharmacia Biotech).

## Results

### Spectrum of HE1/NPC2 mutations

Sequencing of all five exons of *HE1/NPC2* and of their boundaries was performed on genomic DNA isolated from all patients except proband 3, who was included in the initial, "*HE1*" study (Naureckiene et al. 2000; case NPC2-99-04). All 14 new mutant alleles were characterized. As shown in table 3, only five different mutations were identified—two nonsense mutations, E20X and E118X; a 1-bp deletion, 27delG, leading to early termination of protein; a missense mutation, S67P; and a splice mutation, IVS2+5G→A, in the consensus sequence of the 5' donor site of intron 2. The homozygous status of the mutation in probands 7 and 8 was confirmed by sequencing of genomic DNA of both parents. In two independent experiments, the electrophoretic profile of reverse-transcriptase PCR (RT-PCR) products from RNA isolated from proband 8 showed multiple bands (fig. 1). RT-PCR products therefore were subcloned, and 32 subclones were analyzed by sequencing. This analysis revealed 30 abnormally spliced cDNAs and 2 normally spliced cDNAs. Among the 30 mutant cDNAs, only three classes were observed—cDNA with a deletion of the last 76 bp of exon 2 (in 15/30), cDNA with a total deletion of exon 2 (in 6/30), and cDNA with a 10-bp insertion between exons 2 and 3 (in 9/30). The 10-bp



**Figure 1** Multiple abnormal *HE1/NPC2* mRNAs in patient 8—PAGE analysis of RT-PCR products and schematic representations of samples of *HE1/NPC2* cDNA isolated from the patient's fibroblasts.

insertion (gtgcataaaa) corresponds to the 5' end of intron 2 (fig. 1).

#### Western Blot Studies of *HE1/NPC2* in Patients with *NPC2*

*HE1/NPC2* was studied in cultured fibroblasts from a normal subject and from patients with the S67P, IVS2+5G→A, and E20X mutations. As shown in figure 2, no detectable protein was observed in fibroblasts with either IVS2+G→A or E20X, whereas S67P protein appeared as a weak single band—instead of as a doublet, as observed in normal fibroblasts.

#### The E20X Nonsense Mutation—a Frequent Mutant Allele in Patients with *NPC2*

The most striking finding was that E20X was a highly recurrent mutation, observed in five of the eight patients included in the study. Interestingly, the ethnic origin of the patients was quite varied (i.e., Italian, French, Algerian, and Czech). Because the E20X mutation creates a *StuI* restriction site, a simple screening test was devised. Whereas the normal allele remains uncut (325 bp), the mutant allele generates two shorter bands (177 bp and 148 bp) (fig. 3). Parents of probands 1, 3, and 6 were studied by this test, confirming the homozygous status of the mutation in these patients. The study of skin fibroblasts from the abortus also provided ultimate confirmation of four prenatally diagnosed affected siblings in two families. In the eight families studied, the allele frequency of E20X amounted to 56%.

#### Prenatal Diagnosis by Uncultured Chorionic-Villous Sampling in a Family with E20X

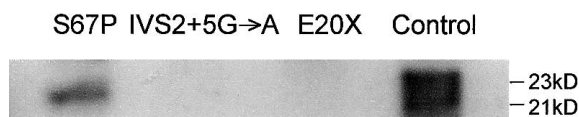
In family 1, which had a homozygous E20X mutation, the third pregnancy was monitored by study of genomic DNA extracted from uncultured chorionic-villus sam-

pling by the *StuI* restriction-enzyme test described above. The fetus was found to be affected, and the pregnancy was terminated at 11 wk gestation. The diagnosis was later confirmed on cultured chorionic-villus samples, by the conventional biochemical strategy (Vanier et al. 1992).

#### Genotype/Phenotype Correlations

Six of the eight patients with *NPC2* who were included in the study had a short life span; four (cases 1–4) did not survive longer than ~6 mo, owing to respiratory or hepatic failure, and the remaining two (cases 5 and 6) developed a neurological disease and died at age 19 mo and 4 years, respectively. In all six of these patients, the *HE1/NPC2* mutation was obviously very deleterious, leading to early termination of the *HE1/NPC2* protein (cases 1–3 and 6, E20X or frameshift; case 4, E118X). All four unrelated patients homozygous for the recurrent E20X mutation presented with hepatosplenomegaly and lung involvement, and three of the four presented with a neonatal cholestatic icterus. Two patients died, of respiratory failure, at age <6 mo, whereas two lived longer and developed a severe neurological disease with onset during infancy. Somewhat unexpectedly, the two affected sibs with a homozygous S67P missense mutation also showed a severe clinical course. Both had marked splenomegaly and less-severe hepatomegaly. The first-born child died early, of respiratory failure, and the other child (case 7), also showing severe respiratory problems, already had obvious neurological involvement by age 18 mo.

Conversely, the two affected sisters in family 8 suffered a quite different disease course, with onset of neurological symptoms during late childhood, slow progression of the disease with long survival, and no prominent pulmonary involvement. The splice mutation underlying the disease in this family was found to produce multiple



**Figure 2** Western blot of HE1/NPC2 in cultured fibroblasts from three patients with NPC2 and from one normal subject. The patients with NPC2 were homozygous for the indicated mutations.

mRNAs, with very small amounts (2/32 clones) of normal message, which may explain the definitely milder disease course in these patients.

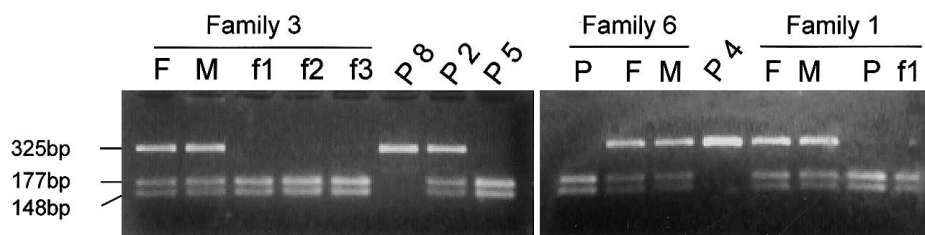
## Discussion

To date, *HE1/NPC2* mutations have been studied in nine unrelated families with NPC2. All mutant alleles have been identified. In the original study establishing *HE1* as the gene underlying NPC2, three mutant alleles corresponded to the E20X nonsense mutation, and one allele corresponded to a 1-bp deletion leading to a frameshift (Naureckiene et al. 2000). The present study, of seven additional families, has led to the identification of only four novel mutations—comprising one nonsense mutation (E118X), one frameshift (27delG), one missense mutation (S67P), and one splice mutation (IVS2+5G→A)—as well as to the finding of 7/14 E20X alleles (table 3). E20X, with an overall allele frequency of 56%, was thus established as the common mutant allele in patients with NPC2. The variable ethnic origin of the families suggests that a founder effect is unlikely. Five of the six mutations thus far described lead to either premature termination of the protein or severe alterations of the transcripts, and the single missense mutation shows a clearly diminished amount of HE1/NPC2 (table 1 and fig. 2). The situation observed with *HE1/NPC2* thus is in strong contrast with the mutational pattern described for *NPC1*—for which a wide variety of mutations have been documented, a majority of which are missense mutations (Carstea et al. 1997; Greer et al. 1999; Yamamoto et al. 2000; Millat et al. 2001; Ribeiro et al. 2001; Sun et al. 2001). Of the missense mutations, several have been shown to result in an essentially normal amount of NPC1 (Yamamoto et al. 2000; Millat et al. 2001; Ribeiro et al. 2001).

From a clinical point of view, the classic signs and symptoms of NPC were present in all cases. Yet, in the population of patients studied, the distribution of the various clinical subtypes was very different from that observed in NPC1. In the latter, patients with a juvenile onset of neurological symptoms constitute ~50% of the cases, patients with an onset of neurological symptoms during infancy constitute ~20%, and patients dying, of liver or respiratory failure, during the 1st year of life, constitute, at most, 10% (Vanier et al. 1991; Vanier

2000). A first remarkable feature of the patients with NPC2 was pronounced lung involvement, which was prominent in six of the seven families for which detailed clinical data were available (Pin et al. 1990; Schofer et al. 1998; Elleder et al. 2001; table 1) and which led to early death in six patients. The possible elective lung involvement in patients with NPC2 has been described elsewhere (Vanier et al. 1996; Schofer et al. 1998). Nevertheless, this feature is not specific to NPC2. Although the complementation group of some published cases with severe lung involvement has not been reported (Kovesi et al. 1996), we have recently definitively shown one patient who died, of acute respiratory failure, at age 2.5 mo, to belong to the NPC1 complementation group (G.M. and M.T.V., unpublished data). Interestingly, similar to the situation for neonatal cholestatic icterus and neurological disease in families with NPC1 (Vanier et al. 1991; Millat et al. 2001), the study of family 7 indicates that fatal respiratory failure and a neurological course can be seen in the same sibship. A second remarkable observation is that, in three of the four families in which patients lived long enough to have neurological symptoms, the neurological disease showed onset during infancy and a rapid course of decline.

The E20X mutation represents a null allele, because all but the signal sequence of HE1/NPC2 is deleted. This logically results in a severe disease phenotype, with all homozygous patients dying at age <4 years. As discussed above, neonatal respiratory manifestations were frequent and could lead to early death at age <6 mo and, thus, before onset of neurological symptoms. Patients who had less-acute or no neonatal systemic manifestations showed an onset of neurological symptoms at age 1–2 years and did not survive past early childhood, as is the rule in the infantile-neurological-onset form (Vanier and Suzuki 1996; Vanier 2000). The 27delG and E118X mutations are also associated with severe disease and thus are likely to represent null alleles. It is difficult to predict a priori the impact of missense mutations. On the basis both of the neurological symptoms already observed, at age 18 mo, in case 7 and of the early death of her brother, it appears that the S67P mutation markedly affects the function of HE1/NPC2. This missense mutation is a nonconservative (polar-to-hydrophobic) substitution that generates a proline, an imino acid known to be particularly important for protein folding. The low level of S67P protein detected by western blot analysis suggests that, in addition to having a possibly deleterious effect on function, this mutation may also affect biosynthesis and/or stability. The IVS2+5G→A mutation is associated with a milder clinical course, since the two siblings homozygous for this mutation both showed a juvenile onset of the neurological disease and a prolonged survival. A more detailed study showed that this splice mutation generated



**Figure 3** Restriction analysis of E20X. E20X creates a *StuI* restriction site. For families 1, 3, and 6 (tables 1 and 3), besides the proband (P) or affected fetuses (f), the study included the father (F) and the mother (M). Lane P5 corresponds to case 5. Proband with other *HE1/NPC2* mutations (cases 8 and 4 [lanes P8 and P4, respectively]) showed a normal pattern and served as controls.

multiple abnormal mRNAs (fig. 3), a situation that has been described in other lysosomal diseases (Ohno and Suzuki 1988). In fibroblasts, a very small proportion (2/32) of correctly spliced transcripts also was demonstrated (fig. 3). Although this was not sufficient to produce enough *HE1/NPC2* for detection by our western blot assay (fig. 2), the presence of low levels of functional protein presumably accounts for the milder clinical course. The question of whether different tissues could show a variability in the level of abnormally/normally spliced RNA transcribed from the IVS2+5G→A mutation also can be raised. Such a situation has been shown to occur in patients with cystic fibrosis who carry the 3,849+10-kb C→T mutation (Rave-Harel et al. 1997; Chiba-Falek et al. 1998) and has been considered as the basis of disease variability.

One potential explanation for the overall very severe clinical and mutational profile of the currently known families with *NPC2* is that genetic complementation studies might have been preferentially performed in patients with a clinical presentation similar to that of the original case (Steinberg et al. 1994). Although this, in some respects, is true of earlier studies (Vanier et al. 1996), in recent years genetic complementation tests have been performed in a quite systematic way, in the laboratories of two of the authors (A.H.F. and M.T.V.) of the present article. Remarkably, the last three identified families with *NPC2* (families 1, 6, and 7) still showed the most common phenotype. Severe alterations of cholesterol trafficking were present in six of the eight families reported in the present study, including the family with a missense mutation. A lesser degree of lysosomal cholesterol storage and impairment of LDL-induced cholesterol esterification was present in cells from patient 4 (Schofer et al. 1998) and in cells from the two siblings in family 8. Yet, no patient with the very mild impairment of cholesterol trafficking that we have described as the “variant” biochemical phenotype (Vanier et al. 1991) has been shown to belong to the *NPC2* complementation group. In fibroblasts from these patients, alterations are demonstrated only after loading with pure LDL (Vanier et al. 1991;

Sun et al. 2001). We agree with Sun et al. (2001) that genetic complementation tests using filipin staining are difficult and not always reliable in patients with the variant phenotype. In consideration of the fact that no *NPC1* mutations could be demonstrated in several of their variant NPC cell lines, Sun et al. (2001) suggested that such patients were good candidates for a systematic screening for *HE1/NPC2* mutations. In our view, excluding the presence of the most frequent *NPC1* mutant alleles—I1061T, P1007A, and G992W/G992R—prior to *HE1/NPC2* sequencing is the proper strategy in newly diagnosed patients with variant NPC. In our survey of 53 variant cell lines, at least one allele carrying any of these four mutations was found in 31 cases. To date, complete sequencing of *HE1/NPC2* cDNA from 10/22 remaining cell lines was achieved, and no abnormality was detected (G.M. and M.T.V., unpublished data). With regard to systematic genetic testing of patients with “classic” NPC (i.e., with severe cholesterol-trafficking alterations), whether to test both for I1061T, on *NPC1*, as we had proposed elsewhere (Millat et al. 1999), and for E20X, on *HE1/NPC2*, should be considered before genetic complementation analysis is performed.

Proper identification of patients with *NPC2* and their molecular characterization are important for two reasons. First, it has an essential, practical impact on genetic counseling; we have shown that the mutational approach greatly improves prenatal testing, resulting in significantly earlier diagnosis. Second, identification of more *HE1/NPC2* mutations may provide important new information about functional domains of *HE1/NPC2* and advance the understanding of its exact function and of its potential interactions with *NPC1*.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for complete genomic DNA sequence of human *HE1* [accession number AC005479] and *HE1/NPC2* [accession number Q15668])  
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for NPC [MIM 257220 and MIM 601015])

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## Critical role for glycosphingolipids in Niemann-Pick disease type C

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**Niemann-Pick type C (NPC) disease is a cholesterol lipidosis caused by mutations in NPC1 and NPC2 gene loci [1]. Most human cases are caused by defects in NPC1 [2], as are the spontaneously occurring NPC diseases in mice [3] and cats [4]. NPC1 protein possesses a sterol-sensing domain [1–3] and has been localized to vesicles that are believed to facilitate the recycling of unesterified cholesterol from late endosomes/lysosomes to the ER and Golgi [1, 5–7]. In addition to accumulating cholesterol, NPC1-deficient cells also accumulate gangliosides and other glycosphingolipids (GSLs), and neuropathological abnormalities in NPC disease closely resemble those seen in primary gangliosidosis [1, 8–12]. These findings led us to hypothesize that NPC1 may also function in GSL homeostasis [8]. To evaluate this possibility, we treated murine and feline NPC models with *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of glucosylceramide synthase, a pivotal enzyme in the early GSL synthetic pathway [13, 14]. Treated animals showed delayed onset of neurological dysfunction, increased average life span (in mice), and reduced ganglioside accumulation and accompanying neuropathological changes. These results are consistent with our hypothesis and with GSLs being centrally involved in the pathogenesis of NPC disease, and they suggest that drugs inhibiting GSL synthesis could have a similar ameliorating effect on the human disorder.**

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### Results and discussion

Niemann-Pick type C disease is widely viewed as a disorder of cholesterol transport and homeostasis [2, 3, 15–18], but substantial evidence also exists for significant involvement of GM2 and GM3 gangliosides and other GSLs [8–11]. In order to evaluate the contribution of GSLs to NPC pathogenesis, we treated NPC-affected mice and cats with an inhibitor of glucosylceramide synthase, the enzyme responsible for the conversion of glucosylceramide to lactosylceramide, a critical early step in the synthesis of gangliosides. We provided NB-DNJ [13, 14] mixed in ground chow to mice (1200 mg/kg/day, beginning at 3.5 weeks of age) and by direct oral administration twice daily to cats (as given below). An assessment of the clinical phenotype of mice with NPC ( $n = 11$ ) versus wild-type littermates ( $n = 9$ ) revealed that in the early stages of the disease, up to 49 days of age, NPC mice did not display evidence of neurological dysfunction and were indistinguishable from wild-type littermates (Figure 1a). However, between 50 and 62 days of age, 78% of untreated NPC mice ( $n = 9$ ) could be classified as having a clinical phenotype consisting of abnormal head and body tremors on movement (tremors of intention) and a pronounced ataxic gait. In contrast, we found that only 11% of NPC mice receiving NB-DNJ ( $n = 9$ ) displayed this clinical phenotype at this age (Figure 1a). Between 63 and 75 days of age, all of the untreated mice (100%) displayed tremors and ataxia, with none living past this time period, whereas only 56% of the treated NPC mice displayed abnormal motor function during this same period (Figure 1a,c). We continued to follow a group of treated mice ( $n = 5$ ) from 76 to 105 days of age, during which time 80% eventually developed the clinical phenotype (Figure 1a).

We also administered NB-DNJ to cats with ( $n = 3$ ) and without ( $n = 4$ ) NPC disease. Compiled data from untreated NPC cats ( $n = 10$ ) from this colony revealed that intention tremor appears at  $9.5 \pm 0.3$  weeks (average  $\pm$  SEM), mild ataxia at  $14.5 \pm 0.3$  weeks, and ataxia with falling at  $21.5 \pm 1.6$  weeks. We initiated treatment of the first NPC cat at 21 weeks of age and administered NB-DNJ for 54 days over an 84 day period, during which time the dose was tapered from 150 mg/kg/day to 50 mg/kg/day. This animal showed a delay in the progression of intention tremor (which was present at the time that treatment was initiated) and in the onset of ataxia with falling. We subsequently treated two younger NPC cats with 50 mg/kg/day of drug beginning at 7 and 13 weeks of age and continuing without interruption for 23 and 56 days, respectively. The younger animal failed to develop any

# Critical role for glycosphingolipids in Niemann-Pick disease type C

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**Niemann-Pick type C (NPC) disease is a cholesterol lipidosis caused by mutations in NPC1 and NPC2 gene loci [1]. Most human cases are caused by defects in NPC1 [2], as are the spontaneously occurring NPC diseases in mice [3] and cats [4]. NPC1 protein possesses a sterol-sensing domain [1–3] and has been localized to vesicles that are believed to facilitate the recycling of unesterified cholesterol from late endosomes/lysosomes to the ER and Golgi [1, 5–7]. In addition to accumulating cholesterol, NPC1-deficient cells also accumulate gangliosides and other glycosphingolipids (GSLs), and neuropathological abnormalities in NPC disease closely resemble those seen in primary gangliosidoses [1, 8–12]. These findings led us to hypothesize that NPC1 may also function in GSL homeostasis [9]. To evaluate this possibility, we treated murine and feline NPC models with *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of glucosylceramide synthase, a pivotal enzyme in the early GSL synthetic pathway [13, 14]. Treated animals showed delayed onset of neurological dysfunction, increased average life span (in mice), and reduced ganglioside accumulation and accompanying neuropathological changes. These results are consistent with our hypothesis and with GSLs being centrally involved in the pathogenesis of NPC disease, and they suggest that drugs inhibiting GSL synthesis could have a similar ameliorating effect on the human disorder.**

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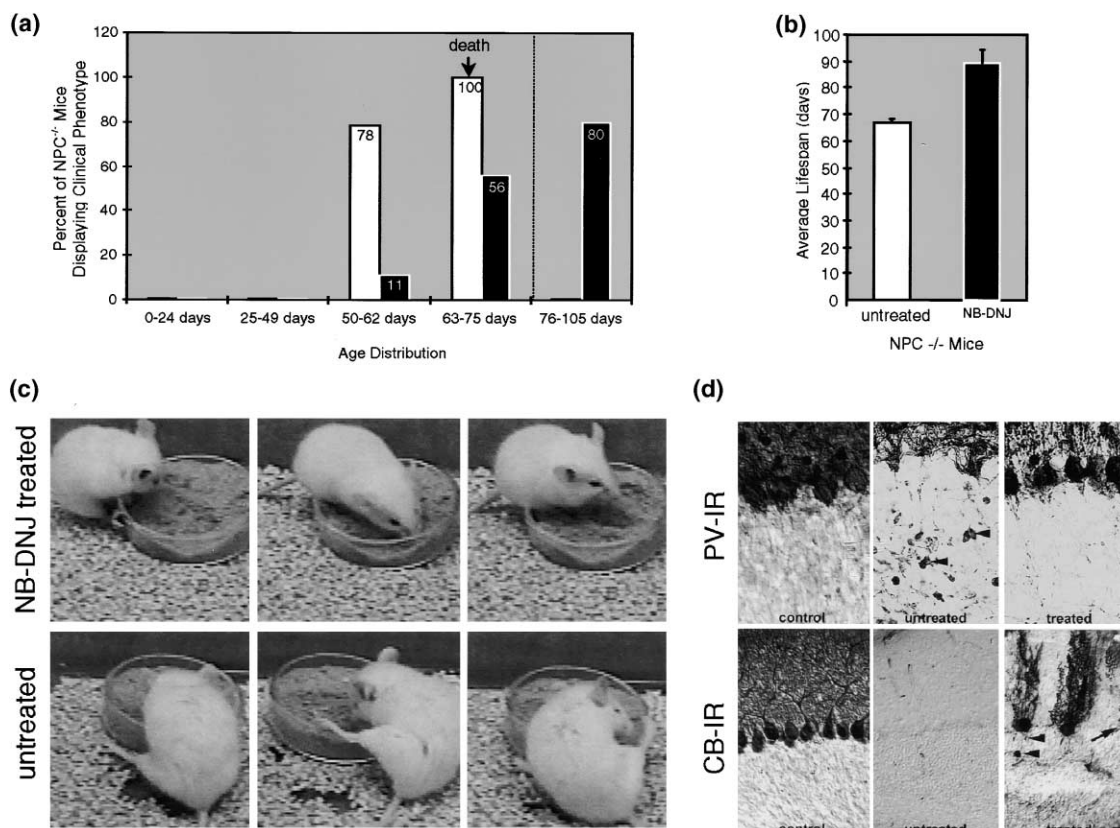
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## Results and discussion

Niemann-Pick type C disease is widely viewed as a disorder of cholesterol transport and homeostasis [2, 3, 15–18], but substantial evidence also exists for significant involvement of GM2 and GM3 gangliosides and other GSLs [8–11]. In order to evaluate the contribution of GSLs to NPC pathogenesis, we treated NPC-affected mice and cats with an inhibitor of glucosylceramide synthase, the enzyme responsible for the conversion of glucosylceramide to lactosylceramide, a critical early step in the synthesis of gangliosides. We provided NB-DNJ [13, 14] mixed in ground chow to mice (1200 mg/kg/day, beginning at 3.5 weeks of age) and by direct oral administration twice daily to cats (as given below). An assessment of the clinical phenotype of mice with NPC (n = 11) versus wild-type littermates (n = 9) revealed that in the early stages of the disease, up to 49 days of age, NPC mice did not display evidence of neurological dysfunction and were indistinguishable from wild-type littermates (Figure 1a). However, between 50 and 62 days of age, 78% of untreated NPC mice (n = 9) could be classified as having a clinical phenotype consisting of abnormal head and body tremors on movement (tremors of intention) and a pronounced ataxic gait. In contrast, we found that only 11% of NPC mice receiving NB-DNJ (n = 9) displayed this clinical phenotype at this age (Figure 1a). Between 63 and 75 days of age, all of the untreated mice (100%) displayed tremors and ataxia, with none living past this time period, whereas only 56% of the treated NPC mice displayed abnormal motor function during this same period (Figure 1a,c). We continued to follow a group of treated mice (n = 5) from 76 to 105 days of age, during which time 80% eventually developed the clinical phenotype (Figure 1a).

We also administered NB-DNJ to cats with (n = 3) and without (n = 4) NPC disease. Compiled data from untreated NPC cats (n = 10) from this colony revealed that intention tremor appears at  $9.5 \pm 0.3$  weeks (average  $\pm$  SEM), mild ataxia at  $14.5 \pm 0.3$  weeks, and ataxia with falling at  $21.5 \pm 1.6$  weeks. We initiated treatment of the first NPC cat at 21 weeks of age and administered NB-DNJ for 54 days over an 84 day period, during which time the dose was tapered from 150 mg/kg/day to 50 mg/kg/day. This animal showed a delay in the progression of intention tremor (which was present at the time that treatment was initiated) and in the onset of ataxia with falling. We subsequently treated two younger NPC cats with 50 mg/kg/day of drug beginning at 7 and 13 weeks of age and continuing without interruption for 23 and 56 days, respectively. The younger animal failed to develop any

**Figure 1**

NB-DNJ treatment in NPC mice and cats was found to delay the onset of clinical neurological disease, to increase longevity (murine NPC), and to reduce cellular pathology in the cerebellum. **(a)** The majority of untreated NPC mice showed a well-defined clinical phenotype (ataxia and intention tremor) between 50 and 62 days of age; by 63–75 days of age, clinical neurological disease and lethality were observed in all untreated NPC mice (open bars). In contrast, far fewer NB-DNJ-treated mice showed the clinical phenotype, and all were viable within this time frame (solid bars). NPC mice treated with NB-DNJ lived to 76–105 days of age, with many of them developing the clinical phenotype during this period. **(b)** An evaluation of longevity in NB-DNJ-treated versus untreated NPC mice showed that treated animals lived to  $89 \pm 5$  days (average  $\pm$  SEM) (solid bar) versus untreated mice, which lived to  $67 \pm 1$  day (open bar). **(c)** Time lapse photographs of an 11-week, 5-day-old NPC mouse treated with NB-DNJ (upper panels) reveals a normally functioning motor system since the animal maintains itself in a stable position on its feeding

dish. In contrast, a 2-week-younger, untreated NPC mouse (9 weeks, 5 days old) is unable to maintain a stable feeding position, falls to the right, and then lurches to the left (lower panels). **(d)** NB-DNJ treatment also reduced the characteristic cerebellar pathology that occurs in NPC disease. The cerebellar cortex from normal mice shows typical distribution of Purkinje cells and layering when it is stained with antibodies to Parvalbumin (upper left panel) and Calbindin (lower left panel). Untreated NPC mice (upper middle panel) display numerous, Parvalbumin-positive axonal spheroids of varying sizes in the granule layer (arrowheads) and regions in which all Calbindin-positive Purkinje cells have disappeared (lower middle panel). Parvalbumin staining in treated NPC mice revealed Purkinje cell axons in the granule cell layer that were mostly devoid of spheroids (upper right panel) and the presence of numerous surviving Calbindin-labeled Purkinje cells (lower right panel). The scale bar (lower right panel) represents 15  $\mu$ m (applies to all).

motor system dysfunction during its treatment period, and the older animal failed to develop ataxia. The four normal cats treated with NB-DNJ (for 19, 23, 54, and 144 days, respectively) generally weighed less at the end of the treatment period than age-matched, untreated, normal cats but otherwise appeared healthy. NB-DNJ not only delayed the onset and/or progression of clinical deterioration in mice and cats but also extended the life span of mice ( $n = 10$ ) from  $67 \pm 1$  day (untreated) to  $89 \pm 5$  days (treated), representing a 25% increase in longevity (Figure 1b).

Neuropathological changes in NPC disease closely resemble those observed in primary ganglioside storage diseases [9, 12, 19]; such changes include the presence of ectopic dendrites on cortical pyramidal neurons and axonal spheroids on cerebellar Purkinje cells and other GABAergic neurons. The cerebellum is severely impacted in both the mouse and cat models of NPC and likely gives rise to a significant portion of the clinical phenotype in these models as well as in human disease [9, 12]. To evaluate the effectiveness of NB-DNJ therapy on cerebellar pathology, we used antibodies to Parvalbumin and Calbindin, respec-

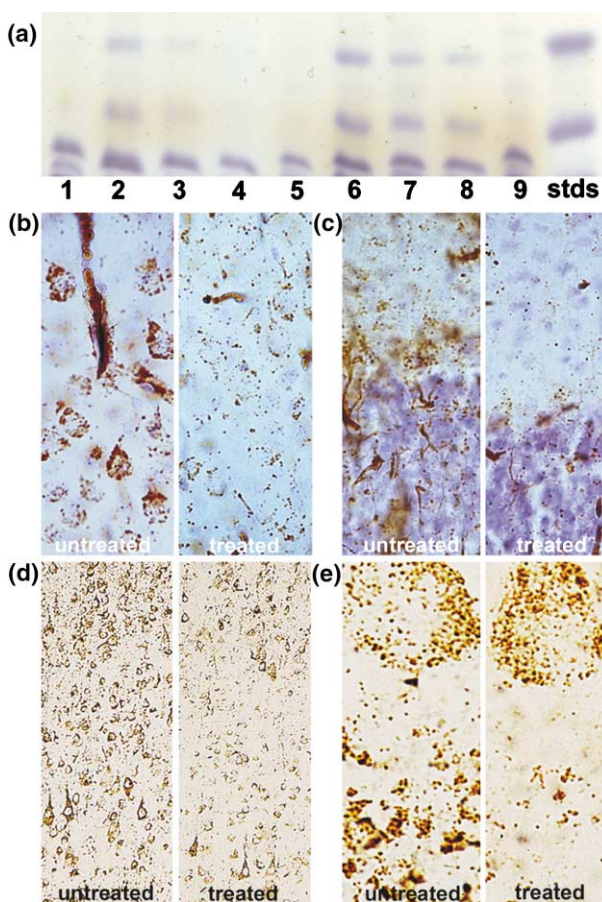


tively, to evaluate axonal spheroid formation and Purkinje cell loss. In wild-type mice and cats, Parvalbumin-immunoreactivity was detected in Purkinje cells and in the molecular layer, while Calbindin staining was observed in Purkinje cells and their dendrites (Figure 1d). In feline NPC disease, we previously found that axonal spheroids initially appeared within the neuropil of deep cerebellar nuclei and adjacent white matter and later spread proximally toward Purkinje cell somata prior to the death of these neurons [12]. Purkinje cell axonal spheroids and death correlate with worsening motor system dysfunction in both the cat and mouse models [12, 19]. Consistent with these earlier studies, untreated 9- to 10-week-old NPC mice in the present study displayed an extensive number of Parvalbumin-positive axonal spheroids (Figure 1d). In contrast, *NB*-DNJ-treated NPC mice at this same age showed diminished spheroid formation in the granule cell layer and preserved Purkinje cell architecture to varying degrees throughout the extent of cerebellar folia. Calbindin staining of untreated NPC mice revealed a nearly complete absence of Purkinje cells in many areas of the cerebellum, while NPC mice treated with *NB*-DNJ exhibited persistence of many of these neurons (Figure 1d).

To assess the effectiveness of *NB*-DNJ in reducing ganglioside storage in NPC brain, we performed high-performance, thin-layer chromatography (HPTLC) [20] and ganglioside immunocytochemistry [9]. HPTLC analysis of NPC-affected brains confirmed GM3 and GM2 ganglioside accumulation in the cerebral cortex of mice and cats with NPC (Figure 2a). *NB*-DNJ treatment resulted in a diminution in ganglioside levels, with notable decreases of GM3 and GM2 gangliosides in the cerebral cortex of both models of NPC (Figure 2a). Semiquantitative analysis of the plates revealed that in feline and murine NPC, respectively, GM3 was reduced from 5.3% to 3.0% and from 6.3% to 4.0% of total lipid bound sialic acid, while GM2 levels were reduced from 2.7% to 1.1% and from 4.2% to 2.2% of total lipid bound sialic acid. Previous studies in which we used immunocytochemical staining to localize GM2 ganglioside in NPC disease revealed that this ganglioside was elevated in neurons of the cerebral cortex, cerebellum, and other brain regions [9]. For the present study, we made a similar analysis in treated versus untreated NPC mice (Figure 2b,c) and cats (Figure 2d,e) to determine if *NB*-DNJ treatment reduced ganglioside storage in these populations of neurons. We found that treatment caused a visible reduction in storage material within individual neurons of the cerebral cortex (Figure 2b,d) and cerebellum (Figure 2c,e) in most sections analyzed. In the cerebral cortex, GM2 immunoreactivity was most conspicuously diminished in pyramidal neurons, while in the cerebellum, GM2 was predominantly reduced in granule cells (mouse, cat) and in Purkinje cells (mouse).

Our demonstration that *NB*-DNJ ameliorates clinical neu-

**Figure 2**



*NB*-DNJ treatment diminishes ganglioside storage in the brains of murine and feline NPC-affected animals. **(a)** HPTLC analysis of brain gangliosides in cats (lanes 1–4) and mice (lanes 5–9) with and without *NB*-DNJ treatment. The standards (stds) lane shows the location of GM3 and GM2 gangliosides (upper and lower spots, respectively). Untreated and treated 33-week-old wild-type cats (lanes 1 and 4, respectively), and 9.5-week-old mice (lanes 5 and 9, respectively) exhibit little or no GM3 and GM2 gangliosides, whereas age-matched, untreated cats and mice with NPC (lanes 2 and 6, respectively) show increased levels of both of these gangliosides. In comparison, the 33-week-old NPC cat treated with *NB*-DNJ for 54 days (lane 3) and the two 9.5-week-old NPC mice treated for 6 weeks (lanes 7 and 8) exhibited reduced levels of both GM3 and GM2 gangliosides. **(b–e)** Representative examples of (b,d) cerebral cortex and (c,e) cerebellum immunostained for GM2 ganglioside in NPC (b,c) mice and (d,e) cats with and without *NB*-DNJ treatment. Tissues are from the same animals used in the HPTLC studies. Untreated NPC mice and cats display GM2 accumulation in pyramidal neurons of the cerebral cortex and in Purkinje and granule cells of the cerebellum. Tissues from animals treated with *NB*-DNJ show reduced levels of GM2 immunoreactivity. The scale bar in the lower right panel represents 100  $\mu\text{m}$  for (d), 10  $\mu\text{m}$  for (e), and 42  $\mu\text{m}$  for (b) and (c).

rological disease in murine and feline NPC underscores the importance of glycosphingolipid metabolism and storage in the NPC disease cascade and suggests that the inhibition of glycosphingolipid synthesis represents an

effective approach toward therapy for this disorder. Interestingly, similar approaches directed at lowering cholesterol in NPC disease were reported to have no significant impact on clinical neurological progression [21, 22]. NB-DNJ therapy has also recently been shown to effectively reduce levels of GM1 ganglioside in circulating leukocytes in human Gaucher disease [23] as well as GM2 ganglioside levels and cytopathology in murine Tay-Sachs and Sandhoff diseases [24, 25]. Substrate deprivation has also been achieved genetically by a cross of Sandhoff disease mice (in which GM2 ganglioside accumulation occurs secondarily to a deficiency in lysosomal  $\beta$ -hexosaminidase) with another line of mice in which the gene for  $\beta$ 1,4-*N*-acetyl-galactosaminyltransferase (GalNAcT, or GM2 synthase) had been ablated [26]. The latter mice lack the ability to produce GM2 and higher-order complex gangliosides. The double mutant animals, in comparison to the mice with Sandhoff disease alone, exhibited significantly less intracellular storage, improved neurological function, and increased longevity. The above findings indicate that substrate deprivation is an effective therapeutic approach to reducing ganglioside accumulation in a number of storage disorders. Recently, NPC mice have also been subject to substrate deprivation through a similar genetic cross with GalNAcT knockout mice [27]. The double homozygous mutants, as anticipated, did not display any detectable accumulation of GM2 or other complex gangliosides but rather possessed higher-than-normal levels of GM3 and GD3 gangliosides. Interestingly, neurons in these animals were reported to lack evidence for an accumulation of unesterified cholesterol, a finding consistent with the possibility that cholesterol storage in NPC neurons occurs secondarily to the accumulation of GM2 or higher-order glycosphingolipids. In contrast to the Sandhoff-GalNAcT double knockouts, clinical disease in the NPC-GalNAcT double mutants did not improve; these results suggest that the storage of higher-order gangliosides and cholesterol are not essential for the generation of brain dysfunction. Notably, it has been previously shown by HPTLC analysis that neutral glycolipids in the early glycosphingolipid pathway (glucosylceramide and lactosylceramide) are also elevated in NPC disease [8, 10, 11], and of these, lactosylceramide remained equivalent to NPC-affected brain in the double mutant mice. It is therefore likely that the treatment of NPC disease with NB-DNJ in our study is beneficial because it diminishes glycosphingolipid synthesis at an early point in the biosynthetic pathway and thus has the potential to reduce multiple storage elements of the disease. It will be important in future studies to determine the precise influence of NB-DNJ on these neutral glycolipids as well as on cholesterol storage.

In summary, we have shown that the glucosyltransferase inhibitor, NB-DNJ, is effective in reducing ganglioside accumulation, cellular pathology, and clinical neurological progression in murine and feline NPC disease. While it is conceivable that the beneficial effect of NB-DNJ is

unrelated to its effect on GLS synthesis, a similar result stemming from the use of a related but more specific glucosylceramide synthase inhibitor, *N*-butyl-deoxygalactonojirimycin, or NB-DGJ (14), on NPC mice (Gondré-Lewis and S.U.W., unpublished data) supports the role for GSLs. Our results are consistent with the view that NPC1 is in some manner linked to ganglioside trafficking or synthesis and that GSLs are centrally involved in the NPC pathogenic cascade. Successful amelioration of NPC disease in murine and feline models also suggests that inhibitors of GSL synthesis may be useful in the clinical management of human NPC disease.

## Materials and methods

### Reagents and models

NB-DNJ (OGT 918; Vevesca) was generously provided by Oxford GlycoSciences. Anti-GM2 ganglioside antibody (1:2,000) was kindly provided by P. Livingston (Memorial Sloan Kettering Medical Center, New York). Anti-Parvalbumin antibody (1:5,000 and 1:10,000 on cortical and cerebellar sections, respectively) and anti-Calbindin antibody (1:500) were purchased from Sigma (St. Louis, Missouri, USA). The BALBc/NPC<sup>nh</sup> mice [11] used to establish a breeding colony were obtained from Dr. Peter Pentchev at the NIH (Bethesda, Maryland, USA), while cats with NPC [28] were from a colony of animals maintained at Colorado State University. For phenotype assessment, mice were evaluated based on the presence or absence of standardized clinical features: The presence of both an ataxic gait and an intention tremor was scored as displaying a clinical phenotype. Two separate trials in mice were done. The first was not blinded; the second trial was blinded and supported the results of the first trial. An assessment of the neurological states of the cats utilized standard postural, reflex, and cranial nerve evaluations performed by a veterinary neurologist lacking knowledge of the treatment status of the animals. Procedures for which animals were used were performed in accordance with the recommendations and approval of the Institutional Animal Care Use Committees of the Albert Einstein College of Medicine and Colorado State University.

### HPTLC and Immunocytochemistry

For HPTLC studies, frozen tissue was homogenized, and total lipids were obtained by standard Folch extraction. A volume of total lipid extract equivalent to 10 mg wet weight was base partitioned via published methods [20]. The upper phase was purified by SEP PAK C<sub>18</sub> chromatography, dried under N<sub>2</sub>, and resuspended, and the extract was separated on HPTLC plates. Total lipid bound sialic acids (which include gangliosides) were imaged with resorcinol and identified by comparison with purified ganglioside standards. Developed HPTLC plates were scanned with an AGFA desktop scanner; HPTLC semiquantitative analysis was done with NIH Imaging 1.62. GM3 and GM2 levels fell within a linear range when compared to a calibration curve determined by a scan of known quantities of GM1 ganglioside. Immunocytochemical methods used in these studies were carried out as previously reported [9].

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## Safety of Parenteral Hydroxypropyl $\beta$ -Cyclodextrin

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**Abstract** Post-treatment data were collected on a patient who received intravenous hydroxypropyl  $\beta$ -cyclodextrin in a dose of 1.5 g/kg in 1985. Although no untoward effects were observed in this patient, rarely occurring agitation and pulmonary edema have been noted after injections into rabbits and dogs, respectively. These complications are analyzed here on the basis of symptoms and on the effects of hydroxypropyl  $\beta$ -cyclodextrin on the biochemistry of a representative lipid, cholesterol, which was studied in rats. It is hypothesized that these untoward effects of parenteral hydroxypropyl  $\beta$ -cyclodextrin are due to complex formation with lipid mediators of pathological responses, of which prostaglandins are one example. These mediators normally have brief and localized functions; if hydroxypropyl  $\beta$ -cyclodextrin happens to be injected when these mediator systems are activated, their influence and the responses of the organism may be increased.

### Introduction

Conjugation of cyclodextrins into their hydroxypropyl ethers by a reaction of low selectivity removes some of the problems of cyclodextrins in pharmaceutical uses, namely low water solubility and parenteral toxicity.<sup>1-4</sup> Consequently, with hydroxypropyl cyclodextrin mixtures, one loses full advantage of the formation of inclusion complexes (host-guest complexes), and lipophilic drugs can be dissolved and made freely and rapidly accessible for absorption into the organism.<sup>5-7</sup> Hydroxypropyl cyclodextrins have very low toxicity by the parenteral route,<sup>2,8</sup> and no adverse effects have been seen in humans;<sup>9</sup> nevertheless their use is not without occasional distress/agitation in rabbits and pulmonary edema in dogs. In this work we analyze the short-term adverse effects and present a hypothesis to explain them, which also may be helpful for minimizing them. Because these effects have been observed only occasionally, we could not study them directly but addressed them by analyzing symptoms and by studying the effect of hydroxypropyl cyclodextrins on homeostasis and concentrations of lipids. Furthermore, we present systematic documentation of the absence of any complications up to 6 years after the intravenous administration of hydroxypropyl  $\beta$ -cyclodextrin to humans.<sup>6</sup>

### Experimental Section

**Materials**—Hydroxypropyl  $\beta$ -cyclodextrin was purchased from Pharmacia Pharmacia Inc., Alachua, FL, Batch 080-32-248. [<sup>14</sup>C]Acetic acid was purchased from Moravia Pharmaceuticals, Inc., Brea, CA, and had a specific activity of 55 mCi/mmol. [<sup>14</sup>C]Cholesterol was purchased from New England Nuclear, Inc., Boston, MA, and had specific activity of 84 mCi/mmol.

**De Novo Synthesis of Cholesterol**—We followed an established procedure.<sup>10</sup> Male rats, F344/D strain, were used in the experiments. The average weight of a rat was 325 g in experiment A and 357 g in experiment B. Animals had unlimited access to food (open formula rat and mouse ration, NIH 07) and to water. Treatment was by

intravenous injection with hydroxypropyl  $\beta$ -cyclodextrin dissolved in isotonic phosphate buffered saline; doses used are in Table I.

In Experiment A, the treatment was performed on the first day at 10 a.m. and 4 p.m., and on the second and third days only at 10 a.m. One hour after the last treatment, an injection of sodium [<sup>14</sup>C]acetate was administered intraperitoneally, 75  $\mu$ Ci per kg of body weight. Rats were sacrificed 1 h after the radiolabel was administered, and the plasma was processed as follows. At first the radioactivity in the plasma was measured after adding an aliquot of the plasma (25 or 50  $\mu$ L) to the aqueous hydrochloric acid (0.5 mL, 0.5 M). To this sample, Ready Gel (3 mL) was added, and radioactivity was measured by liquid scintillation counting. The newly synthesized cholesterol in the plasma was measured by adding an aliquot (1  $\mu$ L) to the solution of aqueous potassium hydroxide (0.5 mL, 10% w/v) and ethanol (2 mL). Cholesterol esters then were saponified at 70 °C for 80 min under argon gas. After the mixture was cooled to room temperature, the total cholesterol was extracted with hexane (three times, 1 mL); the combined extracts were evaporated, the residue was dissolved in benzene (0.5 mL), and radioactivity determined by liquid scintillation counting. Background corrected counts of extracts from rats injected with phosphate buffered isotonic saline were averaged and the counts of all experiments, after correction for background, were expressed as a percentage of the above average, which was assigned a value 100%. The newly synthesized cholesterol in solid tissues was measured after prolonged hydrolysis of samples by alcoholic potassium hydroxide (50 mg/mL), together 60 h at room temperature, 40 h at 37 °C, and 2 h at 80 °C. After further dilution of the samples with aqueous ethanol, the cholesterol was extracted with petroleum ether and its radioactivity was determined as above. All the reported data are averages of duplicate samples. Experiment B was performed using the same protocol, but doses of hydroxypropyl  $\beta$ -cyclodextrin were changed, as described in Table I.

**Leitman/Cholesterol Acyltransferase (LCAT) Activity in Serum**—We used slightly modified established procedures.<sup>7</sup> Hydroxypropyl  $\beta$ -cyclodextrin was added to the freshly collected serum (0.1025 M) rat plasma (0.5 mL) up to the stated concentration, and the run was started by the addition of [<sup>14</sup>C]cholesterol (1  $\mu$ Ci) in ethanol (0.05 mL). The addition caused formation of a small amount of gel which was more apparent at higher concentrations of hydroxypropyl  $\beta$ -cyclodextrin. The gel was dispersed by shaking on a vortex mixer, and the mixture was incubated at 37 °C. At the times specified in Figure 1, small aliquots (2–4  $\mu$ L) were withdrawn and spread on a thin-layer chromatography plate (Merkel Silica gel 60). The plate was developed with a mixture of hexane, 1-ethyl ether, and acetic acid (98:16:1). We scraped off the silica gel from the location where the cholesteryl esters were expected, and determined the radioactivity by liquid scintillation counting. All data reported are averages of duplicate samples.

**Hydroxypropyl  $\beta$ -Cyclodextrin Used in Humans**<sup>6</sup> The endomeration of  $\beta$ -cyclodextrin with propylene oxide was performed using aqueous sodium hydroxide (12.4% w/w) as a solvent and product was purified by extraction with acetone to remove nonpolar impurities; and by a dialysis against distilled water. The average degree of substitution by 253 Cf mass spectrometry was 5.6 (no  $\beta$ -cyclodextrin was detectable in this spectrum) and 1.8 by nuclear magnetic resonance.<sup>9,10</sup> The distribution of substituents on glucose residues was, in molar percentages, as follows: 8, 12.3; 32, 13.6; 33, 5.7; 54, 15.5; 52, 2.4; 52, 6, 6.3; 53, 6, 2.8; 52, 4, 6, 3.0 (measured after hydrolysis by nuclear magnetic resonance).<sup>11</sup> The content of monoglyceride glycol was 0.03% w/w; and of diglyceride glycol was 0.02% w/w.<sup>12</sup>

### Results

**Regulation of Biosynthesis and Esterification of Cholesterol**—*De novo* biosynthesis of cholesterol from me-

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# Safety of Parenteral Hydroxypropyl $\beta$ -Cyclodextrin

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**Abstract** □ Post-treatment data were collected on a patient who received intravenous hydroxypropyl  $\beta$ -cyclodextrin in a dose of 1.5 g/kg in 1985. Although no untoward effects were observed in this patient, rarely occurring agitation and pulmonary edema have been noted after injections into rabbits and dogs, respectively. These complications are analyzed here on the basis of symptoms and on the effects of hydroxypropyl  $\beta$ -cyclodextrin on the biochemistry of a representative lipid, cholesterol, which were studied in rats. It is hypothesized that these untoward effects of parenteral hydroxypropyl  $\beta$ -cyclodextrin are due to complex formation, with lipid mediators of pathological responses, of which prostaglandins are one example. These mediators normally have brief and localized functions; if hydroxypropyl  $\beta$ -cyclodextrin happens to be injected when these mediator systems are activated, their influence and the responses of the organism may be increased.

## Introduction

Conversion of cyclodextrins into their hydroxypropyl ethers by a reaction of low selectivity reduces some of the problems of cyclodextrins in pharmaceutical uses, namely low water solubility and parenteral toxicity.<sup>1-4</sup> Consequently, with hydroxypropyl cyclodextrin mixtures, one takes full advantage of the formation of inclusion complexes (host-guest complexes), and lipophilic drugs can be dissolved and made freely and rapidly accessible for absorption into the organism.<sup>2-4</sup> Hydroxypropyl cyclodextrins have very low toxicity by the parenteral route,<sup>2,5</sup> and no adverse effects have been seen in humans;<sup>6</sup> nevertheless their use is not without occasional distress/agitation in rabbits and pulmonary edema in dogs. In this work we analyze the short-term adverse effects and present a hypothesis to explain them, which also may be helpful for minimizing them. Because these effects have been observed only occasionally, we could not study them directly but addressed them by analyzing symptoms and by studying the effect of hydroxypropyl cyclodextrins on homeostasis and conversions of lipids. Furthermore, we present systematic documentation of the absence of any complications up to 8 years after the intravenous administration of hydroxypropyl  $\beta$ -cyclodextrin to humans.<sup>6</sup>

## Experimental Section

**Materials**—Hydroxypropyl  $\beta$ -cyclodextrin was purchased from Pharmatec/Pharmos Inc., Alachua, FL, Batch 069-32-24A. [<sup>14</sup>C]Acetic acid was purchased from Moravsek Biochemicals, Inc., Brea, CA, and had a specific activity of 56 mCi/mmol. [<sup>14</sup>C]Cholesterol was purchased from New England Nuclear, Inc., Boston, MA, and had specific activity of 52 mCi/mmol.

**De Novo Synthesis of Cholesterol**—We followed an established procedure.<sup>7</sup> Male rats, HSD/SD strain, were used in the experiments. The average weight of a rat was 325 g in experiment A and 337 g in experiment B. Animals had unlimited access to food (open formula rat and mouse ration, NIH 07) and to water. Treatment was by

intravenous injection with hydroxypropyl  $\beta$ -cyclodextrin dissolved in isotonic phosphate buffered saline; doses used are in Table I.

In Experiment A, the treatment was performed on the first day at 10 a.m. and 4 p.m., and on the second and third days only at 10 a.m. One hour after the last treatment, an injection of sodium [<sup>14</sup>C]acetate was administered intraperitoneally, 78  $\mu$ Ci per kg of body weight. Rats were exsanguinated 1 h after the radiolabel was administered, and the plasma was processed as follows. At first the radioactivity in the plasma was measured after adding an aliquot of the plasma (25 or 50  $\mu$ L) to the aqueous hydrochloric acid (0.5 mL, 0.5 M). To that sample, Ready-Gel (8 mL) was added, and radioactivity was measured by liquid scintillation counting. The newly synthesized cholesterol in the plasma was measured by adding an aliquot (1 mL) to the solution of aqueous potassium hydroxide (0.5 mL, 50% w/w) and ethanol (2 mL). Cholesterol esters then were saponified at 70 °C for 80 min under argon gas. After the mixture was cooled to room temperature, the total cholesterol was extracted with hexane (three times, 3 mL), the combined extracts were evaporated, the residue was dissolved in benzene (0.1 mL), and radioactivity determined by liquid scintillation counting. Background corrected counts of extracts from rats injected with phosphate buffered isotonic saline were averaged and the counts of all experiments, after correction for background, were expressed as a percentage of the above average, which was assigned a value 100%. The newly synthesized cholesterol in solid tissues was measured after prolonged hydrolysis of samples by alcoholic potassium hydroxide (50 mg/mL), together 80 h at room temperature, 40 h at 37 °C, and 2 h at 80 °C. After further dilution of the samples with aqueous ethanol, the cholesterol was extracted with petroleum ether and its radioactivity was determined as above. All the reported data are averages of duplicate samples. Experiment B was performed using the same protocol, but doses of hydroxypropyl  $\beta$ -cyclodextrin were changed, as described in Table 1.

**Lecithin:Cholesterol Acyltransferase (LCAT) Activity in Serum**—We used slightly modified established procedure.<sup>8</sup> Hydroxypropyl  $\beta$ -cyclodextrin was added to the freshly collected citrated (0.0025 M) rat plasma (0.5 mL) up to the stated concentration, and the run was started by the addition of [<sup>14</sup>C]cholesterol (1  $\mu$ Ci) in ethanol (10  $\mu$ L). The addition caused formation of a small amount of gel which was more apparent at higher concentrations of hydroxypropyl  $\beta$ -cyclodextrin. The gel was dispersed by shaking on a vortex mixer, and the mixture was incubated at 20 °C. At the times specified in Figure 1, small aliquots (2–4  $\mu$ L) were withdrawn and spotted on a thin-layer chromatography plate (Merck Silica gel 60). The plate was developed with a mixture of hexane, diethyl ether, and acetic acid (83:16:1). We scraped off the silica gel from the location where the cholesteryl esters were expected, and determined the radioactivity by liquid scintillation counting. All data reported are averages of duplicate samples.

**Hydroxypropyl  $\beta$ -Cyclodextrin Used in Human**<sup>6</sup>—The condensation of  $\beta$ -cyclodextrin with propylene oxide was performed using aqueous sodium hydroxide (12.4% w/w) as a solvent and product was purified by extraction with acetone (to remove nonpolar impurities) and by a dialysis against distilled water. The average degree of substitution by 252 Cf mass spectrometry was 5.6 (no  $\beta$ -cyclodextrin was detectable in this spectrum) and 4.9 by nuclear magnetic resonance.<sup>9,10</sup> The distribution of substituents on glucose residues was, in molar percentages, as follows: S<sub>0</sub>, 42.8; S<sub>2</sub>, 13.6; S<sub>3</sub>, 5.7; S<sub>6</sub>, 15.5; S<sub>2</sub>, 3, 5.2; S<sub>2</sub>, 6, 6.0; S<sub>3</sub>, 6, 2.6; S<sub>2</sub>, 3, 6, 3.0 (measured after hydrolysis by nuclear magnetic resonance).<sup>11</sup> The content of monopropylene glycol was 0.02% (w/w) and of dipropylene glycol was 0.02% w/w.<sup>11</sup>

## Results

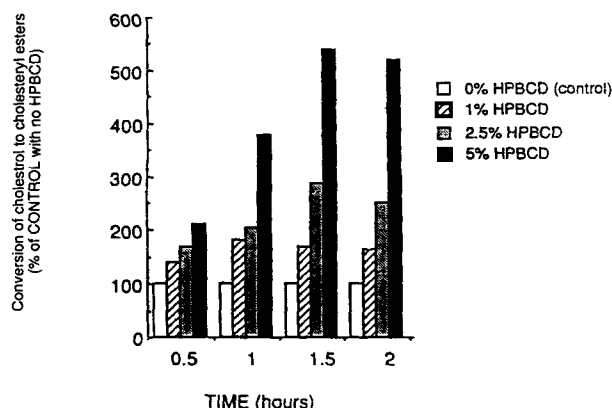
**Regulation of Biosynthesis and Esterification of Cholesterol**—*De novo* biosynthesis of cholesterol from acetic

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**Table 1—Effects of Intravenous Hydroxypropyl  $\beta$ -Cyclodextrin on De Novo Cholesterol Synthesis in Rats**

	Experiment A				Experiment B			
intravenous treatment	4 $\times$ PBS <sup>a</sup>		4 $\times$ 0.5 g HPBCD <sup>b</sup> per kg		3 $\times$ PBS		3 $\times$ 1.5 g HPBCD per kg	
Animal	1	2	3	4	1	2	3	4
De novo cholesterol (cpm/mL of plasma)	391	772	849	590	1433	599	1080	6466
% of averaged controls	67	133	146	102	141	60	106	46
Liver weight (g)	12.5	9.3	8.9	10.9				
% of averaged controls	115	85	82	100				
De novo cholesterol (cpm/g of liver)	887	1415	2011	1699				
% of averaged controls	77	123	175	148				

<sup>a</sup> Phosphate-buffered isotonic saline. <sup>b</sup> Hydroxypropyl  $\beta$ -cyclodextrin.



**Figure 1—Effects of hydroxypropyl  $\beta$ -cyclodextrin (HPBCD) on the conversion of [<sup>14</sup>C]cholesterol into [<sup>14</sup>C]cholesteryl esters in rat plasma.**

acid can be measured easily in the rat, and it is an accurate indicator of the overall availability of cholesterol in an organism.<sup>7</sup> The level of biosynthesis varies with the overall state of the animal, but any such variations are much smaller than the changes routinely observed upon cholesterol depletion in animals (e.g., by bile binding agents in food); these changes may occur over 1 order of magnitude.<sup>7</sup> The results of experiments A and B (Table 1) indicate that a series of intravenous administrations of hydroxypropyl  $\beta$ -cyclodextrin did not greatly change the levels of biosynthesis of cholesterol from those seen in controls.

Another parameter that gives pertinent information on the effects of parenteral hydroxypropyl  $\beta$ -cyclodextrin is the rate of conversion of cholesterol into cholesteryl esters. This process is affected by the rate of transfer of lipids between the lipoproteins through the aqueous phase; the respective measurements can be made *ex vivo* in plasma.<sup>8,12,13</sup> Using sera from the same strain of rats as above, we measured the time dependencies of the conversion (Figure 1). The results show a moderate increase proportional to the concentration of hydroxypropyl  $\beta$ -cyclodextrin added.

**Short-Term Adverse Effects in Animals**—No short-term adverse effects of parenteral hydroxypropyl  $\beta$ -cyclodextrin have been observed in mice and rats in this laboratory (ref 5 and unpublished materials) or recorded in the literature.<sup>2,14</sup> In a rabbit (New Zealand, white), one adverse reaction was observed during the period when about 100 boluses in all were administered to about a dozen animals.<sup>15</sup> The rabbit in question became distressed and agitated immediately after the start of the injection; when the injection was interrupted, the rabbit recovered. Tests for irritation and deviation from isotonicity of the injection solution were negative, and the same rabbit tolerated the solution well when it was administered as eye drops. In dogs, parenteral hydroxypropyl  $\beta$ -cyclodextrin was recorded to cause pulmonary edema in two animals out of three.<sup>16,17</sup> Cardiovascular function was followed closely but was found not to be disturbed in any of the

three dogs;<sup>16,17</sup> thus edema could not be due to defective heart activity. Pathological leakage of liquid into the alveoli was thus a probable culprit. In monkeys, intravenous hydroxypropyl  $\beta$ -cyclodextrins did not cause any adverse effects.<sup>14</sup>

**Absence of Adverse Effect in Humans**—A young boy suffering from severe hypervitaminosis A, probably due to an inherited variance in the metabolism of that vitamin (patient 2, of ref 6), received an infusion of hydroxypropyl  $\beta$ -cyclodextrin in June 1985. The dose was 470 mg/kg/day, a total of 30 g over 4 days in the form of a 5% solution in water. (Characterization of the hydroxypropyl  $\beta$ -cyclodextrin used by the presently available methods is described in the Experimental Section). Clinical observations and those laboratory results which were related to the disease and which were collected at the time of infusion therapy had been published previously;<sup>6,18</sup> the distribution of retinoids in lipoproteins was described later.<sup>15</sup> Table 2 displays the clinical chemistry data documenting the lack of short-term adverse effects of the infusion. The data show that in spite of a badly balanced injection solution, no hemolysis was seen. A number of related derivatives of  $\beta$ -cyclodextrin were shown to have somewhat lower hemolytic activity *in vitro*; the present results indicate that the improvements which occur when these are used instead of hydroxypropyl  $\beta$ -cyclodextrin *in vivo* may not be significant. Studies on animals have shown that intravenous hydroxypropyl  $\beta$ -cyclodextrin causes morphological changes in kidneys which are repaired only slowly. Table 3 shows the clinical chemistry data related to kidney function after the infusion; no damage is seen.

## Discussion

Hydroxypropyl  $\beta$ -cyclodextrins, free or containing complexed drug, equilibrate rapidly with surrounding lipids after entering an organism.<sup>15,19</sup> Thus a new reservoir—a pool of circulating and rapidly accessible lipids—is formed. Formation of this pool increases the total concentration of dissolved lipids, but because these newly dissolved lipids are in complex forms, their physicochemical activities are not changed. Cholesterol is probably the major lipid present in this new pool; this suggestion follows from the observation that it was mainly this lipid which was excreted with hydroxypropyl  $\beta$ -cyclodextrin into urine.<sup>15</sup>

Presently we are interested in learning how formation of such a new pool of cholesterol will affect its biosynthesis; this biosynthesis is regulated on the basis of cholesterol availability or of a preception of availability. Biosynthesis of cholesterol can be measured easily. In a rat it occurs at a basal level; thus both increases and decreases in the conversion of acetic acid to cholesterol can be measured. The present results show that parenteral hydroxypropyl  $\beta$ -cyclodextrin has no strong effects on the biosynthesis of cholesterol; formation of a new circulatory pool of a lipid is not perceived as an excess or a lack of that lipid. Although these results are relevant

Table 2—Clinical Chemistry Data Collected during the Infusion of Hydroxypropyl  $\beta$ -Cyclodextrin into the Patient

	Preinfusion	Infusion of HPBCD				Day after the Infusion		
Day	-1	1	2	3	4	1	2	3
White blood count	6900			8400		7700		
Hemoglobin	11.5			10.5		10.2		
Sodium (mequiv/L)	133				138			
Potassium (mequiv/L)	3.6			4.6				
Chloride (mequiv/L)	107			107				
Bicarbonate (mequiv/L)				21	14		16	
Urea (mmol/L)	2.4	1.7		3.2			3.2	
Creatine ( $\mu$ mol/L)	35			44			35	
Protein (g/L)	56	63	57	63	64	66	65	
Albumin (g/L)	40	41	38	40	40	42	41	
Glucose (mg/dL)	72	104	94		103		113	
Total calcium (mmol/L)	2.00	2.07	1.98	2.07	1.90	2.10	2.05	
Ionized calcium (mmol/L)	1.06	1.14	1.04	1.13	1.05	1.07	1.08	1.09
pH	7.48	7.38	7.46	7.42	7.41	7.45	7.46	7.40
Phosphate (mmol/L)	1.55	1.67	1.71	1.77	1.64	1.80	1.77	
Cholesterol	199	214	187	178	179	209	211	
Uric acid (mmol/L)	0.23	0.25	0.22	0.18	0.15	0.27	0.17	
Alkaline phos (IU/L) (<300)	950	>700	647	>600	611	559	650	
T bilirubin ( $\mu$ mol/L)	29	32	27	29	30	34	29	
Conjugated bilirubin	2	1	2	2	2	2	3	
ALT U/L (<35)	32	43	65	35	36	33	47	
AST U/L (<40)	67	74	62	70	35	55	82	
$\gamma$ GT U/L (50)	52	59	15	54	56	53	55	
LDM U/L (<200)				536				

Table 3—Renal Function Tests of Patient

Date	BUN (mg/dL)	UREA (mM)	Creatine ( $\mu$ M)
May 1984	9	3.2	9
June 11, 1985	6.7	2.4	26
June 12, 1985	4.8	1.7	35
First day of HPBCD infusion			
June 14, 1985	9	3.2	44
Third day of HPBCD infusion			
June 17, 1985	9	3.2	35
Second day after infusion			
October 29, 1990	13.7	4.9	67
April 2, 1991	9.8	3.5	46

strictly to cholesterol, other lipids form analogous new pools, and their biosynthesis is probably also not affected.

Also investigated were the effects of this new pool on phenomena that depend principally on the rates of transfer of lipids through the aqueous phase of circulating blood. Esterification of cholesterol in plasma is such a process because it depends on rates of transfer of lipids between lipoprotein particles.<sup>12,13</sup> Formation of a new, rapidly accessible pool of cholesterol may be expected to increase the transfer, and indeed the rate of conversion was observed to rise.

On the basis of the above results, we can propose a hypothesis to explain the short-term adverse effects seen in animals. The results suggest that although the hydroxypropyl  $\beta$ -cyclodextrin in circulation may not affect the overall balances of the compounds with which it forms complexes, the processes that are controlled by time may be changed. The above-mentioned adverse effects may be caused by a wider distribution of signal lipid mediators of pathological responses; this distribution is controlled principally by the rate of blood flow and the rate of metabolism of the mediators. Some of these mediators are called "local hormones" and are synthesized in many organs in response to specific stimuli. They exert their effects mostly at the site of their synthesis, unlike endocrine hormones.<sup>20</sup> If a new pool of these local hormones is formed through complex formation, they may escape

inactivation through the rapid local metabolism, may be carried to other organs, and may mediate pathological responses there.

Other results also support this hypothesis. Parenteral cyclodextrins and cyclodextrin derivatives were shown previously to assist distribution of foreign toxins. Toxic effects of retinoic acid, injected intraperitoneally, were accelerated by intravenous injections of 2,6-di-*O*-methyl- $\beta$ -cyclodextrin.<sup>21</sup> Subcutaneous injections of  $\beta$ -cyclodextrin aided the cancerogenic effects of *N*-ethyl-*N*-(2-hydroxyethyl)nitrosoamine, which was administered orally.<sup>22</sup> Parenteral cyclodextrins thus may assist in the distribution of externally introduced toxins; largely the same can be expected for potential toxins formed by the organism itself. Furthermore, the above-mentioned lipid mediators, of which the products of oxygenation of arachidonic acid are examples, are known to form complexes with cyclodextrins<sup>23</sup> and thus their distribution in the organism may be expected to be affected by parenteral cyclodextrin derivatives. Our hypothesis is also compatible with the sporadic or species-specific character of the adverse effects. According to the hypothesis, cyclodextrins exert these effects by aggravating the response of the organism to some specific stimuli that may be unrelated to the cyclodextrin administration; therefore these effects are bound to be irregular.

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## Genetic variations and treatments that affect the lifespan of the NPC1 mouse

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**Abstract** Niemann-Pick type C (NPC) disease is a multisystem disorder caused primarily by a mutation in the *npc1* gene. These studies evaluated the effect of genetic background, deletion of additional genes, and administration of several agents on the age at death in a murine model of this disorder. Such factors as differing strain background or genetic drift within a given background in the *npc1*<sup>-/-</sup> mouse significantly altered the age at death and the degree of organ disease. Genetic deletion of *Sint9* (GM3 synthetase) or *Nr1h2* [liver X receptor (LXR)β] shortened the life of the *npc1*<sup>-/-</sup> animals. Daily treatment of the *npc1*<sup>-/-</sup> mice with an LXR agonist or administration of a single dose of cyclodextrin, with or without the neurosteroid allopregnanolone, significantly slowed neurodegeneration and increased the lifespan of these animals. These data illustrate that the age at death of the *npc1*<sup>-/-</sup> mouse can be significantly influenced by many factors, including differences in strain background, other inactivating gene mutations (*Sint9* and *lrxβ*), and administration of agents such as LXR agonists and, particularly, cyclodextrin. It is currently not clear which of these effects is nonspecific or which might relate directly to the molecular defect present in the NPC1 syndrome.—Liu, B., H. Li, J. J. Repa, S. D. Turley, and J. M. Dietschy. Genetic variations and treatments that affect the lifespan of the NPC1 mouse. *J. Lipid Res.* 2008. 49: 663–669.

**Supplementary key words** cyclodextrin • allopregnanolone • neurodegeneration • nuclear receptors • lysosomes • gangliosides • Purkinje cells • Niemann-Pick type C1 disease

Niemann-Pick type C (NPC) disease is one of a number of lysosomal storage diseases and results primarily from a mutation that inactivates the protein NPC1 that is responsible for the movement of unesterified cholesterol from the late endosomal/lysosomal compartment to the cytosol in every cell (1). As a result, cholesterol accumulates in virtually all tissues in the body, causing organ dysfunction that may manifest clinically as hepatosplenomegaly, prolonged neonatal jaundice, liver dysfunction, pulmonary failure, and, ultimately, progressive neurological dysfunction secondary to selective neurodegeneration. These clinical findings are reproduced in a murine model of this disease that also arose as a spontaneous mutation in the *npc1* gene (2, 3). In mice homozygous for this mutation, and with a BALB/c genetic background, the concentration of cholesterol becomes increased with age in nearly every organ, and there is neonatal cholestasis, liver cell death, pulmonary dysfunction, and selective nerve cell death (4–8). Despite this block in intracellular sterol movement, however, an increased rate of cholesterol synthesis within the cytoplasmic compartment allows for essentially normal rates of plasma membrane sterol turnover, bile acid synthesis, and steroid hormone production (9, 10).

This murine model has proved very valuable as an experimental animal in which to explore the effects of various genetic and pharmacological manipulations in an attempt to better understand the pathophysiology of this disorder. The effect of such manipulations can be assessed in these animals by evaluating a number of end points, such as liver function tests, pulmonary diffusion capacity, the level of macrophage infiltration in tissues, mRNA levels for different inflammatory proteins, clinical neurological function, and quantitation of specific nerve cell numbers. Despite the availability of these many precise end points, the age at death of these animals continues to be used as a relatively easy measure of the effects of experimental manipulations that might alter the genetic defect in this disease. However, we recently found that many manipulations, some of which probably do not relate directly to the defect in cholesterol transport, can alter the age at which the *npc1*<sup>-/-</sup> mouse dies. If this is the case, then changes in the age at death could lead to erroneous conclusions with respect to the molecular events dictating the pathophysiology of NPC disease. This report outlines a number of factors affecting the lifespan of the *npc1*<sup>-/-</sup> mouse, including genetic drift in the colony, changes in the strain background of the mutant animals, deletion of the function of an addi-

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Abbreviations: LDLR, low density lipoprotein receptor; LXR, liver X receptor; NPC, Niemann-Pick type C; UTSW, University of Texas Southwestern.

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# Genetic variations and treatments that affect the lifespan of the NPC1 mouse

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**Abstract** Niemann-Pick type C (NPC) disease is a multisystem disorder caused primarily by a mutation in the *npc1* gene. These studies evaluated the effect of genetic background, deletion of additional genes, and administration of several agents on the age at death in a murine model of this disorder. Such factors as differing strain background or genetic drift within a given background in the *npc1*<sup>−/−</sup> mouse significantly altered the age at death and the degree of organ disease. Genetic deletion of *Siat9* (GM3 synthetase) or *Nr1h2* [liver X receptor (LXR)β] shortened the life of the *npc1*<sup>−/−</sup> animals. Daily treatment of the *npc1*<sup>−/−</sup> mice with an LXR agonist or administration of a single dose of cyclodextrin, with or without the neurosteroid allopregnanolone, significantly slowed neurodegeneration and increased the lifespan of these animals. These data illustrate that the age at death of the *npc1*<sup>−/−</sup> mouse can be significantly influenced by many factors, including differences in strain background, other inactivating gene mutations (*Siat9* and *Lxrβ*), and administration of agents such as LXR agonists and, particularly, cyclodextrin. It is currently not clear which of these effects is nonspecific or which might relate directly to the molecular defect present in the NPC1 syndrome.—Liu, B., H. Li, J. J. Repa, S. D. Turley, and J. M. Dietschy. Genetic variations and treatments that affect the lifespan of the NPC1 mouse. *J. Lipid Res.* 2008. 49: 663–669.

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These clinical findings are reproduced in a murine model of this disease that also arose as a spontaneous mutation in the *npc1* gene (2, 3). In mice homozygous for this mutation, and with a BALB/c genetic background, the concentration of cholesterol becomes increased with age in nearly every organ, and there is neonatal cholestasis, liver cell death, pulmonary dysfunction, and selective nerve cell death (4–8). Despite this block in intracellular sterol movement, however, an increased rate of cholesterol synthesis within the cytoplasmic compartment allows for essentially normal rates of plasma membrane sterol turnover, bile acid synthesis, and steroid hormone production (9, 10).

This murine model has proved very valuable as an experimental animal in which to explore the effects of various genetic and pharmacological manipulations in an attempt to better understand the pathophysiology of this disorder. The effect of such manipulations can be assessed in these animals by evaluating a number of end points, such as liver function tests, pulmonary diffusion capacity, the level of macrophage infiltration in tissues, mRNA levels for different inflammatory proteins, clinical neurological function, and quantitation of specific nerve cell numbers. Despite the availability of these many precise end points, the age at death of these animals continues to be used as a relatively easy measure of the effects of experimental manipulations that might alter the genetic defect in this disease. However, we recently found that many manipulations, some of which probably do not relate directly to the defect in cholesterol transport, can alter the age at which the *npc1*<sup>−/−</sup> mouse dies. If this is the case, then changes in the age at death could lead to erroneous conclusions with respect to the molecular events dictating the pathophysiology of NPC disease. This report outlines a number of factors affecting the lifespan of the *npc1*<sup>−/−</sup> mouse, including genetic drift in the colony, changes in the strain background of the mutant animals, deletion of the function of addi-

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tional genes, and treatment with several agents that may alter the natural history of this disease. These studies suggest the potential hazards inherent in interpreting the relevance of various treatments to overcoming this genetic defect based on changes in the age at death of the experimental animals.

## MATERIALS AND METHODS

### Animals

These studies were undertaken using eight groups of genetically modified mice. One group of animals lacking functional NPC1 protein (*npc1*<sup>-/-</sup>) on a BALB/c background was derived from heterozygous animals originally obtained from the National Institutes of Health 9 years ago (2, 3) and maintained in the animal colony at the University of Texas Southwestern (UTSW) Medical School. A second group of similar *npc1*<sup>-/-</sup> mice was derived from heterozygous founders purchased from the Jackson Laboratories (BALB/cNctr-*Npc1*<sup>m1N</sup>/J; stock number 003092) this year. Animals from the original UTSW colony were also crossed with low density lipoprotein receptor-deficient (*ldlr*<sup>-/-</sup>) (11), *Siat9*<sup>-/-</sup> (12), liver X receptor  $\beta$ -deficient (*lxr $\beta$* <sup>-/-</sup>) (13), and *abca1*<sup>-/-</sup> animals (14) to yield double knockouts designated *npc1*<sup>-/-</sup>/*ldlr*<sup>-/-</sup>, *npc1*<sup>-/-</sup>/*Siat9*<sup>-/-</sup>, *npc1*<sup>-/-</sup>/*lxr $\beta$* <sup>-/-</sup>, and *npc1*<sup>-/-</sup>/*abca1*<sup>-/-</sup>, respectively. In each case, the appropriate control *npc1*<sup>-/-</sup> littermates having the same respective, mixed strain backgrounds were derived and are designated *npc1*<sup>-/-</sup>/*ldlr*<sup>+/+</sup>, *npc1*<sup>-/-</sup>/*Siat9*<sup>+/+</sup>, *npc1*<sup>-/-</sup>/*lxr $\beta$* <sup>+/+</sup>, and *npc1*<sup>-/-</sup>/*abca1*<sup>+/+</sup>. In nearly all cases, these various crosses occurred over >10 generations. After these crosses, the female mice were maintained on a low-cholesterol (0.03%, w/w) rodent diet (No. 7002; Harlan Teklad, Madison, WI) during the pregnancies. The *npc1* genotype was determined by a PCR method (2), and mice exhibiting aberrant lifespans relative to similarly treated animals (outliers) had their genotypes confirmed at the time of death. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the UTSW Medical School.

### Treatments

All mice were weaned at 19 days of age and fed ad libitum a low cholesterol (0.02%, w/w) rodent diet (No. 7001; Harlan Teklad) or the ground meal form of this same diet containing either cholesterol (1%, w/w) or an LXR agonist (T0901317; Cayman Chemical). Intake of this latter diet provided each animal with an approximate daily dose of T0901317 equal to 50 mg/kg body weight. Based on a previous publication (15), other groups of mice were administered a single subcutaneous injection at the scruff of the neck at 7 days of age of a 20% (in saline) solution of 2-hydroxypropyl- $\beta$ -cyclodextrin (4,000 mg/kg body weight) with either 5.6 degrees of substitution (Aldrich; product 332607) or 4.5 degrees of substitution (Sigma; product H107). These values indicate the average number of hydroxypropyl groups per cyclodextrin molecule. In addition, in some experiments, allopregnanolone (5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one) (Sigma; product P8887) was added to the cyclodextrin solutions at a concentration of 1.5 mg/ml (to provide 25 mg/kg body weight) as described (15).

### Tissue cholesterol concentration, liver function tests, and cerebellar histology

In one experiment, 56-day-old animals were used to measure the weight and cholesterol concentration in different organs, and plasma was obtained to measure liver function tests (8, 16).

In another experiment, samples of the anterior cerebellum were taken for histological examination.

### Animal monitoring

The general clinical condition of the mice was monitored daily. Once the mice began to show difficulty accessing the pelleted basal diet, they were also provided access to a powdered form of this diet. When mice were no longer able to take food or water, they were humanely euthanized, and this was considered the day of death.

### Statistical analysis

All numeric results are expressed as means  $\pm$  SEM for each treatment group. GraphPad Prism software (GraphPad, San Diego CA) was used to perform all statistical analyses. To compare multiple groups, a one-way ANOVA with Neumann-Keuls post hoc comparison was performed. For comparison of only two groups, a Student's *t*-test was used. In all cases, statistically significant differences were declared at *P* < 0.05.

## RESULTS

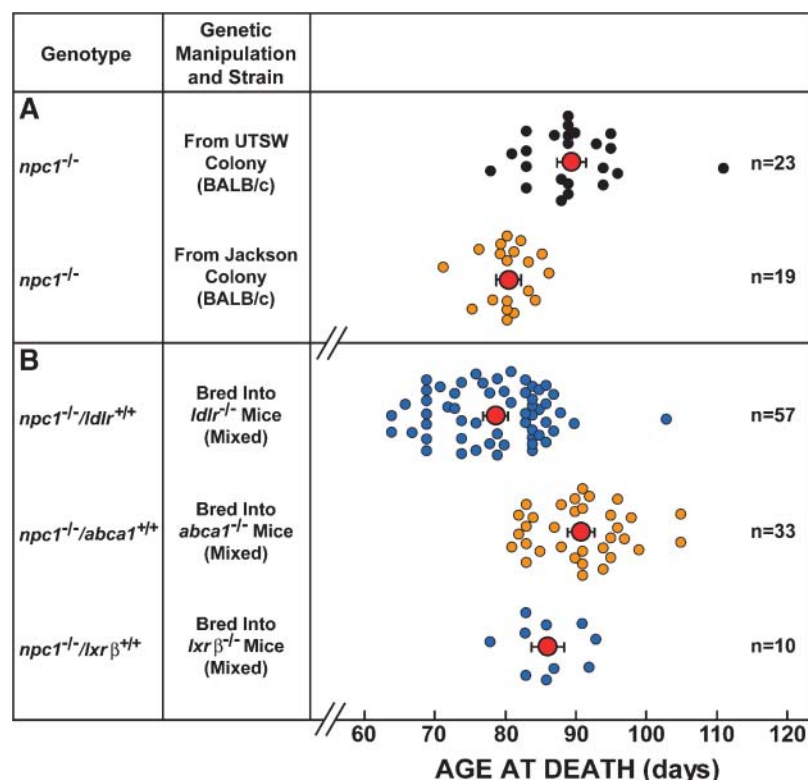
### Effects of genetic drift and strain background on age at death of *npc1*<sup>-/-</sup> mice

Although the UTSW and Jackson colonies of NPC mice were derived from the same source, they had been isolated from one another for  $\sim$ 9 years. Whereas both colonies were maintained on a BALB/c background, *npc1*<sup>-/-</sup> animals from the UTSW colony lived significantly longer (89  $\pm$  1.4 days) than those from the Jackson colony (80  $\pm$  0.8 days) when observed during the same time period (Fig. 1A). However, although both groups of mice had similar body weights (Fig. 2A), enlarged livers (Fig. 2C), and cholesterol concentrations in the liver (Fig. 2D), spleen (Fig. 2E), and lung (Fig. 2F), the plasma transaminase levels were only increased half as much (Fig. 2H, I) in the Jackson, compared with the UTSW, animals. Thus, genetic drift in these two colonies affected both the age at death and the severity of the liver disease in these *npc1*<sup>-/-</sup> mice with the BALB/c background.

Importantly, crossing these animals into different genetic backgrounds also had significant effects on lifespan. For example, introducing the C57BL/6 and 129/SvJ backgrounds from the *ldlr*<sup>-/-</sup> animals into the BALB/c *npc1*<sup>-/-</sup> mice decreased the age at death to 79  $\pm$  1.0 days, whereas the similar, mixed strain background derived from the *abca1*<sup>-/-</sup> mice prolonged lifespan to 91  $\pm$  1.1 days (Fig. 1B). Also of note is the range of values seen in these 142 *npc1*<sup>-/-</sup> mice, where some animals died at 63 days of age but others reached 109 days of age (Fig. 1). Even when the strain background in a group of *npc1*<sup>-/-</sup> animals was constant, the range of ages at death varied over an interval of 20 days in some groups.

### Effects of changes in sphingolipid metabolism and lipoprotein cholesterol flux on age at death

In the studies shown in Fig. 3, every experimental manipulation was judged against an appropriate, untreated *npc1*<sup>-/-</sup> control group with an identical genetic back-



**Fig. 1.** Effects of genetic separation and different strain backgrounds on the age at death of Niemann-Pick type C1-deficient (*npc1*<sup>-/-</sup>) mice. The original *npc1*<sup>-/-</sup> mice on a BALB/c background were obtained directly from the National Institutes of Health in 1997 to establish a colony at the University of Texas Southwestern (UTSW) Medical School. A similar colony was later established at the Jackson Laboratories. A: Age at death of 42 animals taken from these two colonies and observed simultaneously. B: The same *npc1*<sup>-/-</sup> mice with the BALB/c background from the UTSW colony were bred over the last 9 years with low density lipoprotein receptor-deficient (*ldlr*<sup>-/-</sup>; C57BL/6 and 129/Svj backgrounds), *abca1*<sup>-/-</sup> (C57BL/6 and 129/Ola backgrounds), and liver X receptor  $\beta$ -deficient (*lxr\beta*<sup>-/-</sup>; C57BL/6 and 129/Svj backgrounds) mice to generate double knockout animals. This panel shows the age at death of the littermates of these crosses that had only the *npc1*<sup>-/-</sup> genotype but were of different mixed strain backgrounds. The colors have no specific meaning and are used only to separate individual mice in the various groups. The red circles show the mean  $\pm$  SEM for the number (n) of animals with each genetic background.

ground. However, for the purposes of this figure, all of these 142 control *npc1*<sup>-/-</sup> animals were combined into a single group and normalized to a mean age at death of 85 days (Fig. 3A). Although various gangliosides also accumulate in the brains of the *npc1*<sup>-/-</sup> mice (17), elimination of GM3 ganglioside synthesis by deleting the *Siat9* gene significantly decreased the lifespan of the mutant mice (Fig. 3B). In contrast, there was no effect on the age at death of these *npc1*<sup>-/-</sup> animals when they were fed cholesterol or when LDLR activity was deleted (Fig. 3C). In the first instance, increased delivery of cholesterol carried in chylomicrons to the liver worsened the hepatic disease (8), whereas in the second instance, deletion of LDLR activity increased the delivery of cholesterol carried in LDL to the lungs and worsened pulmonary function (16). Neither manipulation, however, is known to alter cholesterol flux across the blood-brain barrier.

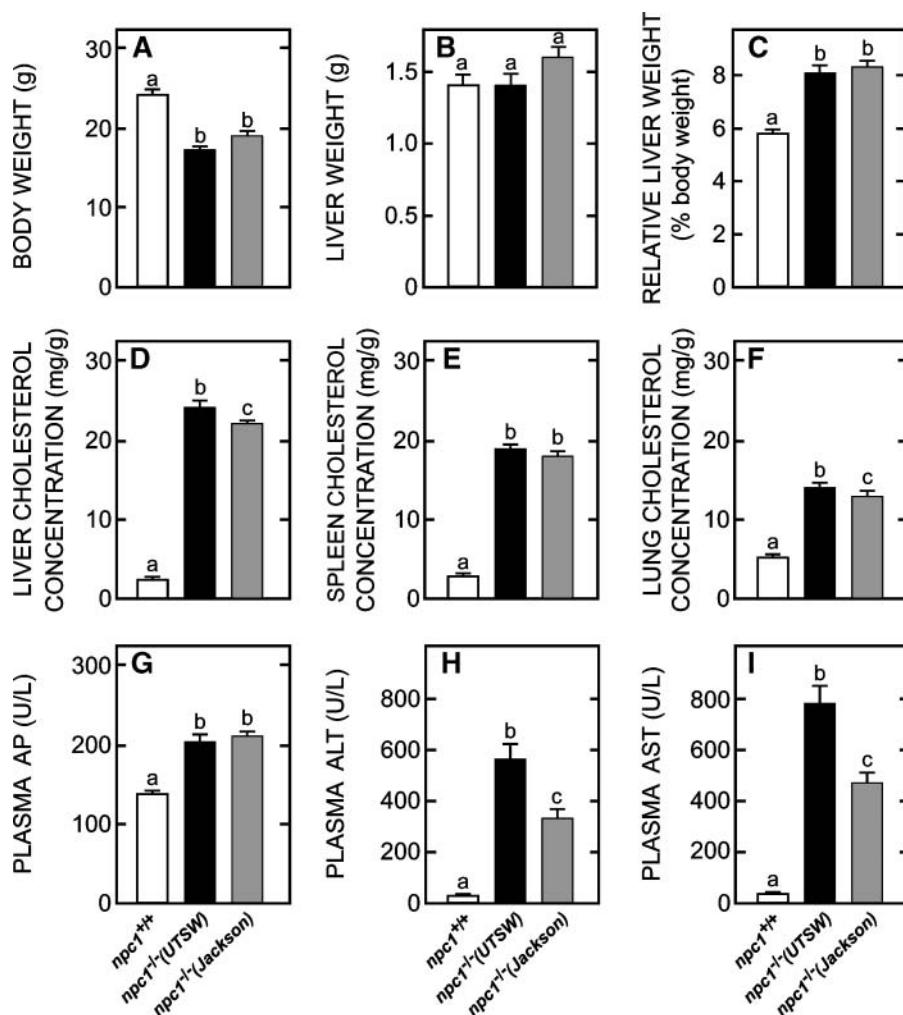
#### Effects of altered LXR function or administration of cyclodextrin on age at death

In contrast to these latter observations, deletion of LXR $\beta$  function shortened lifespan ( $74 \pm 2.2$  days), whereas stimulation of this receptor activity by administration of the agonist significantly prolonged life ( $93 \pm 1.8$  days) (Fig. 3D). Because this receptor is expressed in the brain (18), these effects could conceivably reflect changes in cholesterol flux across the central nervous system. More strikingly, a single dose of cyclodextrin given at 7 days of age markedly prolonged the average life ( $>108$  days) of these *npc1*<sup>-/-</sup> mice, but the addition of allopregnanolone to this regimen had no additional effect (Fig. 3E).

Although there was an  $\sim 80\%$  reduction in Purkinje cell number in the untreated *npc1*<sup>-/-</sup> mice (Fig. 4A, B), treatment with cyclodextrin increased the number of these cells surviving at 49 days of age nearly 3-fold (Fig. 4C).

## DISCUSSION

Although the age at death of the *npc1*<sup>-/-</sup> animal would seem to be a definitive end point to use in evaluating the effectiveness of various manipulations that might elucidate the pathogenesis of NPC disease, it is clear from these studies that many presumably nonspecific manipulations can significantly alter this end point. We found, for example, that offering the partially debilitated mouse ground food or subjecting the animal to daily exercise on a rotarod apparatus significantly prolongs the lifespan of the animal compared with the mouse offered only solid food or not subjected to exercise (data not shown). In addition to these effects of animal husbandry, the genetic background of the mice can have a major effect on the age at death even though the mutation in the NPC1 gene presumably is unchanged. For example, genetic drift in the colony (Fig. 1A) or the introduction of a new genetic background from other mouse strains (Fig. 1B) can significantly change not only the age at death but, in some cases, the severity of the clinical disease (Fig. 2) seen in the *npc1*<sup>-/-</sup> mouse. These observations raise several important issues with respect to performing studies in which age at death is the end point. First, the control *npc1*<sup>-/-</sup> mice against which the effectiveness of a particular manipula-

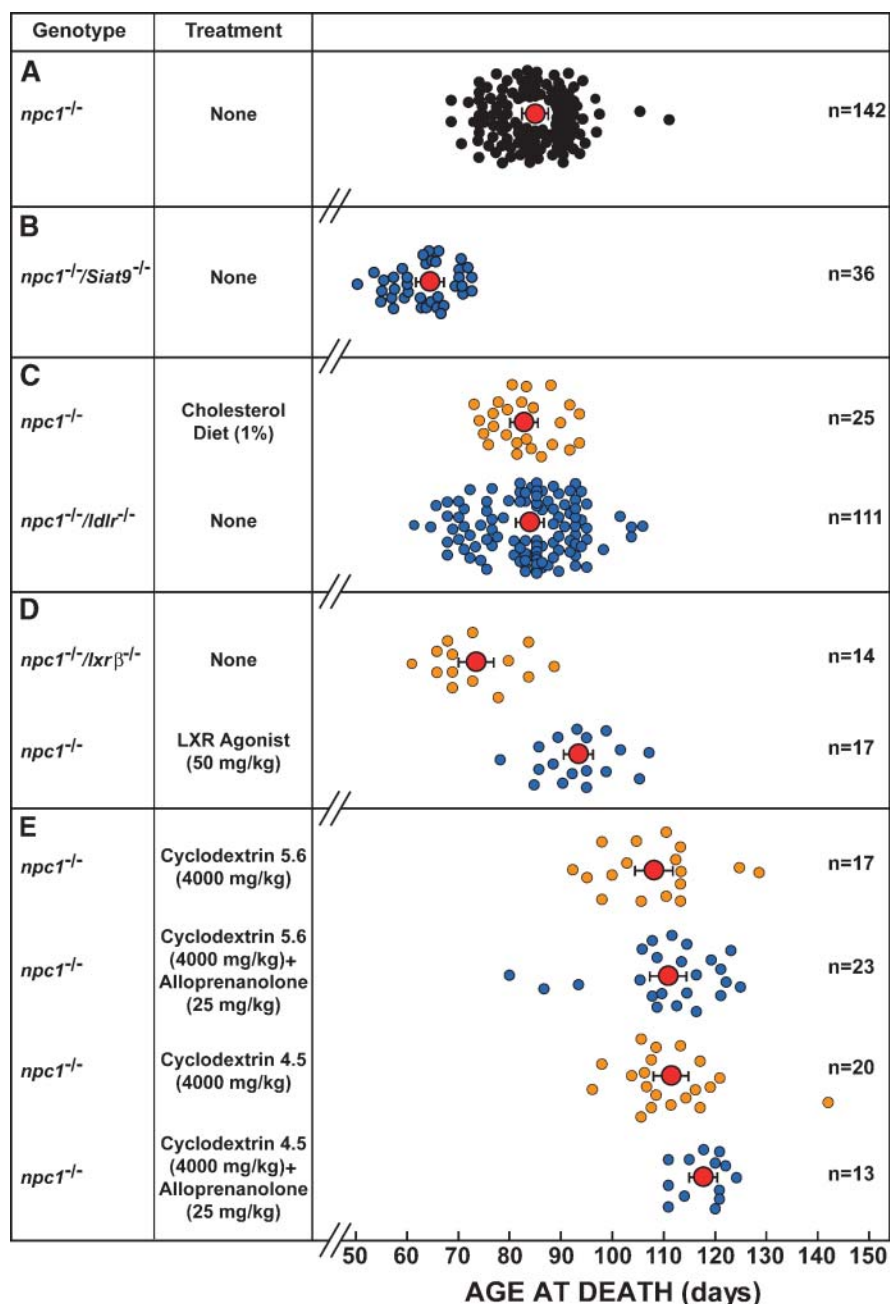


**Fig. 2.** Tissue cholesterol concentrations and liver function tests in mice from the UTSW and Jackson Laboratory colonies. These studies were carried out in 56-day-old *npc1*<sup>+/+</sup> and *npc1*<sup>-/-</sup> mice derived from both the UTSW and Jackson Laboratory colonies. As there were no differences in metabolic parameters in the *npc1*<sup>+/+</sup> animals that came from either colony, these two groups of mice were combined into a single group (*n* = 9). The groups of *npc1*<sup>-/-</sup> mice were derived from either the UTSW (*n* = 8) or Jackson Laboratories (*n* = 8) stocks and studied independently. Whole body animal weights (A) and absolute (B) and relative (C) liver weights were measured, as were the concentrations of cholesterol in the liver (D), spleen (E) and lungs (F). Alkaline phosphatase (AP; G), alanine aminotransferase (ALT; H), and aspartate aminotransferase (AST; I) levels in plasma were also measured. Values depict means  $\pm$  SEM of data from eight or nine mice per group. Bars denoted by different letters are statistically different (*P* < 0.05).

tion is judged must come from the same colony of animals and must have the same genetic strain background as the treated animals. Second, it is important that issues of husbandry, diet, and exercise be identical in the control and experimental groups. Third, even when these conditions are met, the age at death commonly will vary over a range of 20 days or more (Fig. 1). Thus, to avoid misleading results, the control and experimental groups must have relatively large numbers of animals. Finally, even under the best of experimental circumstances, and when the genotype is confirmed, every large group of mice consistently has outliers (Figs. 1, 3) that deviate significantly from the remaining animals in terms of the age at death. Measurements, particularly morphological charac-

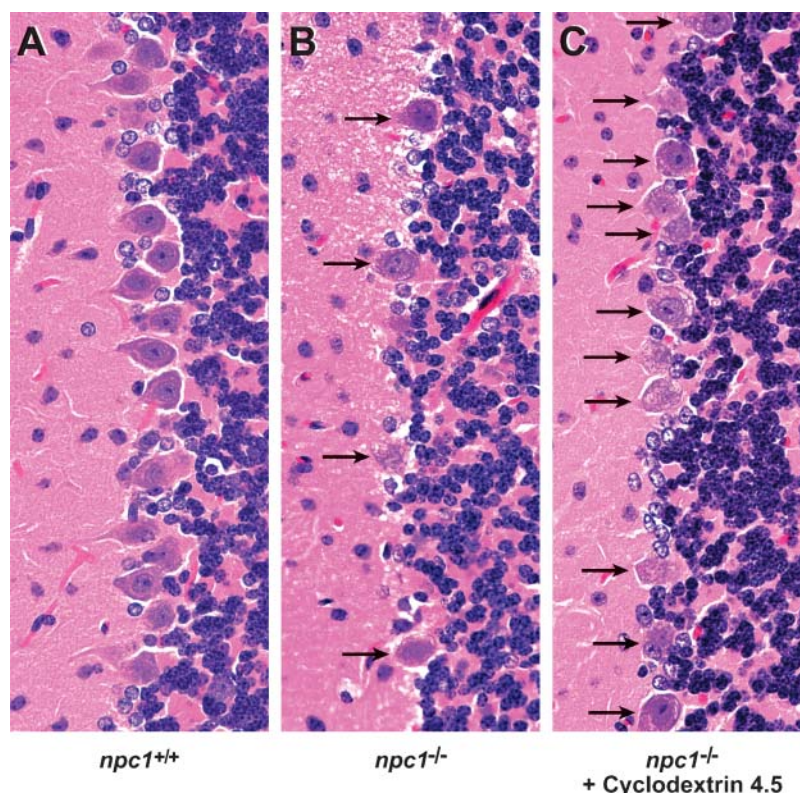
terizations, inadvertently carried out in one or two of these outliers also could lead to serious errors of interpretation.

However, in spite of all of these potential artifacts, other observations suggest that the age at death can be altered significantly by different genetic or pharmacological manipulations that do relate to the underlying transport defect found in the cells of the *npc1*<sup>-/-</sup> mouse. For example, although the accumulation of gangliosides has been postulated to play a role in neuron death in this disorder, inactivation of the gene *Galgt1*, which is responsible for the enzyme-synthesizing GM2 gangliosides, slightly decreases the age at death of the *npc1*<sup>-/-</sup> mouse (69 vs. 79 days) (19), and inactivation of *Siat9*, which encodes the protein responsible for the synthesis of GM3 gangliosides, short-



**Fig. 3.** Effect of various genetic deletions and dietary or pharmacological treatments on the age at death of the *npc1<sup>-/-</sup>* mice. This figure shows the age at death of the *npc1<sup>-/-</sup>* animals after nine different treatments or genetic manipulations. In each case, the treated, experimental animals were compared with untreated, control *npc1<sup>-/-</sup>* mice with the same strain background. For the purposes of this figure, however, the ages at death in all of these groups were normalized so that the control *npc1<sup>-/-</sup>* animals had a mean age at death of 85.0 days. A: Data for these combined 142 control *npc1<sup>-/-</sup>* mice are shown. B: Effects of deleting the activity of the enzyme GM3 synthetase (*Siat9<sup>-/-</sup>*) that is responsible for the synthesis of GM3 gangliosides. C: Effects of either feeding a 1% cholesterol diet from weaning or deleting the LDLR are shown. D: Effects of deleting LXRβ or driving this receptor with an agonist are illustrated. E: Effects of administering a single dose of cyclodextrin 5.6 or cyclodextrin 4.5, with or without alloprenanolone, on the age at death are shown. The colors have no specific meaning and are used only to separate the individual mice in the different groups. The red circles show the mean  $\pm$  SEM for the number (n) of animals in each treatment group. The manipulations in C did not significantly alter the age at death compared with the control animals in A ( $P > 0.05$ ), whereas those treatments in B, D, and E all significantly altered the age at death ( $P < 0.0001$ ).





**Fig. 4.** Histological examination of the cerebellum of *npc1*<sup>-/-</sup> mice treated with cyclodextrin. Cerebella were harvested from animals at 49 days of age, and histological preparations of the anterior portion were stained with hematoxylin and eosin to show Purkinje cells. A: Tissue from *npc1*<sup>+/+</sup> animals injected with saline. B: Tissue from *npc1*<sup>-/-</sup> mice injected with saline. C: Tissue from *npc1*<sup>-/-</sup> animals treated with a single injection of cyclodextrin 4.5 at 7 days of age. The arrows in B and C point to surviving Purkinje cells.

ens the life expectancy of the mutant animals even more (65 vs. 85 days) (Fig. 3B). Similarly, although manipulating the amount of chylomicron cholesterol introduced into the vascular space or the level of LDL-cholesterol clearance has no effect on the age at death of these animals (Fig. 3C), manipulation of the activity of LXR, a receptor known to regulate cholesterol flux across the brain (20), clearly alters the age at death of the *npc1*<sup>-/-</sup> mice (Fig. 3D). Finally, another group of molecules known to alter cholesterol in the plasma membrane of cells (21), the cyclodextrins, markedly prolong the life of the mutant mice, and this effect appears to be independent of the administration of the neurosteroid, allopregnanolone. This latter observation is particularly striking because it follows the administration of the cyclodextrin as a single dose at 7 days of age. Clearly, the molecular and physiological alterations brought about by these changes in sphingolipid and cholesterol metabolism that account for the observed changes in age at death need to be investigated in detail. However, these observations emphasize that any studies that use age at death as a major end point must avoid the potential misleading results that can arise from failure to control the genetic background of the different groups of animals, failure to control different nutritional and other environmental factors affecting the animals, and, most importantly, failure to use adequate numbers of

mice in the experimental groups to obtain a reliable measure of the actual time of death.

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# Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the *npc1*<sup>−/−</sup> mouse

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Niemann-Pick type C disease is largely attributable to an inactivating mutation of NPC1 protein, which normally aids movement of unesterified cholesterol (C) from the endosomal/lysosomal (E/L) compartment to the cytosolic compartment of cells throughout the body. This defect results in activation of macrophages in many tissues, progressive liver disease, and neurodegeneration. In the *npc1*<sup>−/−</sup> mouse, a model of this disease, the whole-animal C pool expands from 2,082 to 4,925 mg/kg body weight (bw) and the hepatic C pool increases from 132 to 1,485 mg/kg bw between birth and 49 days of age. A single dose of 2-hydroxypropyl- $\beta$ -cyclodextrin (CYCLO) administered at 7 days of age immediately caused this sequestered C to flow from the lysosomes to the cytosolic pool in many organs, resulting in a marked increase in cholesterol esters, suppression of C but not fatty acid synthesis, down-regulation of genes controlled by sterol regulatory element 2, and up-regulation of many liver X receptor target genes. There was also decreased expression of proinflammatory proteins in the liver and brain. In the liver, where the rate of C sequestration equaled 79 mg·d<sup>−1</sup>·kg<sup>−1</sup>, treatment with CYCLO within 24 h increased C movement out of the E/L compartment from near 0 to 233 mg·d<sup>−1</sup>·kg<sup>−1</sup>. By 49 days of age, this single injection of CYCLO resulted in a reduction in whole-body C burden of >900 mg/kg, marked improvement in liver function tests, much less neurodegeneration, and, ultimately, significant prolongation of life. These findings suggest that CYCLO acutely reverses the lysosomal transport defect seen in NPC disease.

cholesterol | lysosome | cyclodextrin | liver X receptor

Niemann-Pick type C (NPC) disease results from mutations that inactivate 1 of 2 proteins, either NPC1 (95% of cases) or NPC2 (1, 2). These 2 proteins normally act in concert to facilitate the movement of unesterified cholesterol (C), derived from the cellular uptake of lipoproteins, across the limiting membrane of the lysosome to the metabolically active pool of sterol in the cytosolic compartment (3, 4). As a consequence of these mutations, C progressively accumulates in the late endosomal/lysosomal (E/L) compartment of virtually every cell in the body from the time of early fetal development until death of the child or animal (5). In the *npc1*<sup>−/−</sup> mouse (6), a murine model of the common form of NPC disease, the animal sequesters an amount of sterol equal to 67 mg/kg body weight (bw) every day so that the whole-body sterol pool increases from ~2,200 to 5,700 mg/kg between birth and 7 weeks of age (7). Pathologically, this C accumulation is associated with infiltration of activated macrophages into many organs and with parenchymal cell death. In both the human and the mouse, these histological changes, in turn, result in the clinical syndromes of pulmonary failure, liver dysfunction, and progressive neurological disease (3, 8). Importantly, in a particular organ, the severity of disease is proportional to the amount of C sequestered in that tissue (3, 9, 10).

The sterol that is sequestered in the late E/L compartment of cells in each organ is internalized through 2 different transport mechanisms involving the cellular uptake of lipoproteins through either

receptor-mediated or bulk-phase endocytosis. Receptor-mediated uptake utilizes the low-density lipoprotein receptor (LDLR) that is able to bind lipoproteins containing either apolipoprotein (apo) E or apoB<sub>100</sub>, such as remnants of chylomicrons and very low-density lipoprotein (VLDL), and LDL (11, 12). The velocity of this uptake process in a particular organ *in vivo* is determined by the concentration of lipoproteins in the plasma, the level of LDLR expression, the affinity constant for a given lipoprotein, and the reflection coefficient defining the permeability characteristics of the capillary membranes overlying the parenchymal cells of that tissue. The rate of uptake of lipoproteins by bulk-phase endocytosis is also determined by the concentration of the particles in the plasma and the reflection coefficient of the capillary membranes; however, in addition, it may be influenced by nonsolvent water effects within the endocytic vesicles (13). In the *npc1*<sup>−/−</sup> mouse, 88% of LDL cleared from the plasma and sequestered in different tissues is taken up by receptor-mediated endocytosis, whereas only 12% is cleared by bulk-phase endocytosis (7). However, when LDLR activity is eliminated, as in the *npc1*<sup>−/−</sup>*ldlr*<sup>−/−</sup> animal, all LDL is cleared by bulk-phase endocytosis. Because the rate constants for these two processes are very different in different organs, the profile of C sequestration in tissues varies markedly in the *npc1*<sup>−/−</sup>*ldlr*<sup>+/+</sup> and *npc1*<sup>−/−</sup>*ldlr*<sup>−/−</sup> animals (3). Importantly, these rate constants for lipoprotein uptake by either receptor-mediated or bulk-phase endocytosis are unaffected by the presence or absence of the NPC1 mutation. Whole-animal LDL clearance by receptor-mediated (~520 mL·d<sup>−1</sup>·kg<sup>−1</sup>) and bulk-phase (~62 mL·d<sup>−1</sup>·kg<sup>−1</sup>) endocytosis is approximately the same in *npc1*<sup>+/+</sup> and *npc1*<sup>−/−</sup> mice (7). However, there is evidence that the lipid accumulation seen in *npc1*<sup>−/−</sup> cells does lead to a 2-fold expansion of the bulk-phase volume found within the lysosomal compartment and to a diminished rate of movement of this bulk-phase fluid back out of the cell (14).

One way to take advantage of these observations to control the severity of disease caused by the NPC1 mutation is to reduce the amount of lipoprotein cholesterol available for uptake into cells. Thus, blocking the intestinal absorption of sterol with a drug like ezetimibe, which lowers the amount of C reaching the liver carried in chylomicron remnants, markedly reduces hepatocyte damage and improves hepatic function (9). Increasing sterol excretion out of the brain across the blood-brain barrier, which presumably leaves

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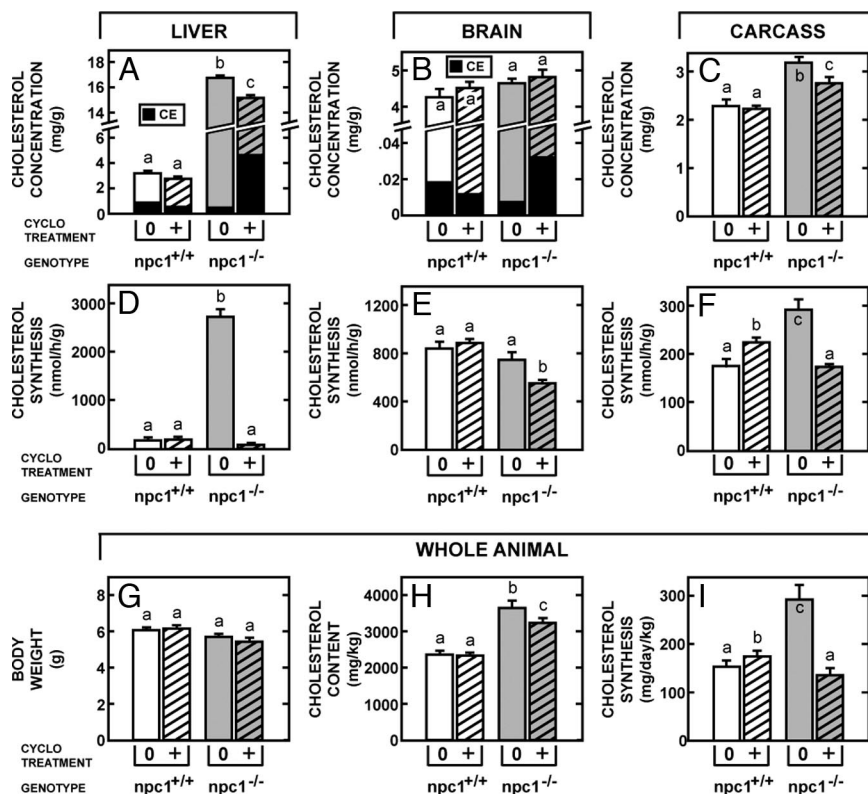
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**Fig. 2.** A single dose of CYCLO 4.5 administered to 7-day-old pups markedly altered sterol metabolism in the *npc1*<sup>-/-</sup> mice 24 h later but had no effect in the *npc1*<sup>+/+</sup> animals. The unesterified and esterified cholesterol concentrations are shown in the liver (A) and brain (B), whereas total cholesterol concentrations are given for the remaining tissues of the carcass (C). (D–F) Cholesterol synthesis rates are shown for the same 3 tissue compartments. These values (G) are combined to give whole-animal cholesterol pools (H) and synthesis rates (I) in these same animals. To obtain these latter values, the rates of incorporation of <sup>3</sup>H<sub>2</sub>O into sterols by the different organs were converted to absolute rates of cholesterol synthesis. Statistically significant differences are indicated by the different letters ( $n = 6$ ,  $P < 0.05$ ).

#### Acute Effects of CYCLO Administration on Major Regulatory Mechanisms in Liver and Brain

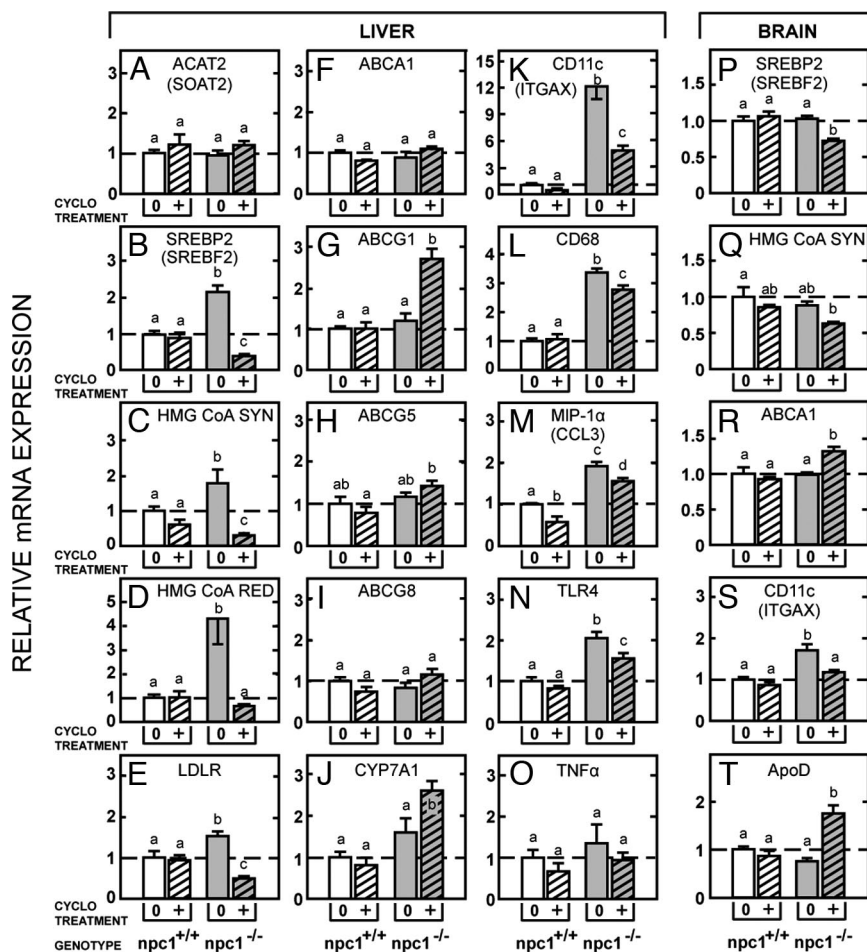
If, in fact, CYCLO treatment had promoted such a sudden efflux of C into the cytosolic compartment, there should be major changes manifested in the 2 regulatory systems in this compartment, sterol regulatory element 2 (SREBP2) and liver X receptor (LXR), known to respond to variations in the size of the metabolically active sterol pool (17, 18). Because sterol O-acyltransferase 2 in the liver is constitutive, relative mRNA levels for this gene were the same in the 4 groups of pups (Fig. 3A). In contrast, mRNA levels for SREBP2 and its target genes, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase I (SYN), HMG CoA reductase (RED), and LDLR, were all significantly elevated in the untreated *npc1*<sup>-/-</sup> mice (Fig. 3B–E). After CYCLO treatment, however, these mRNA levels were markedly suppressed, below even the levels seen in the *npc1*<sup>+/+</sup> pups. In contrast, mRNA expression of the LXR target genes, ATP-binding cassette (ABC) A1, ABCG1, ABCG5, ABCG8, and cytochrome P450 7A1 (CYP7A1), was not elevated in the untreated *npc1*<sup>-/-</sup> mice (Fig. 3F–J). After CYCLO treatment, however, the expression of ABCG1 and CYP7A1 became significantly elevated. Failure to observe a change in ABCA1 mRNA levels in liver is consistent with the report that the hepatic ABCA1 transcript isoform is not under regulatory control of dietary C or LXR (19). Another characteristic feature of NPC disease of the liver is the presence of activated macrophages. This was reflected in elevated mRNA levels of a number of inflammatory factors, including CD11c, CD68, MIP-1 $\alpha$  (chemokine ligand 3), and TLR4 but not TNF- $\alpha$  (Fig. 3K–O). Of note, the mRNA level of TNF- $\alpha$  was elevated in older *npc1*<sup>-/-</sup> mice (20). Treatment with CYCLO also significantly reduced this overexpression. Brain showed similar changes 24 h after CYCLO administration. The relative mRNA level of SREBP2 and HMG CoA SYN was reduced (Fig. 3P and Q), and that of the LXR target genes, ABCA1 and apoD, was significantly increased (Fig. 3R and T). In addition, the elevated mRNA level of CD11c, reflecting activated glia in the brain, was restored to normal after CYCLO treatment (Fig. 3S).

#### Long-Term Effects in the Adult Mouse of CYCLO Administration in Infancy

The next studies were designed to identify the long-term effects, if any, in 49-day-old adult mice that had been administered CYCLO at 7 days of age. Again, CYCLO administration to the *npc1*<sup>+/+</sup> mice had no effect on either C concentrations or synthesis rates in the liver, brain, and other organs (Fig. 4A–C). However, there were marked residual effects in the *npc1*<sup>-/-</sup> animals. Six weeks after administration of CYCLO, when none of the compound was retained in the body, the concentration of C was still significantly reduced in the liver, kidney, spleen, and other organs. No reduction was seen in the brain, however, because most of the C in this organ is present in myelin, so that any changes that might have occurred in neurons are obscured. In addition, in nearly every organ, including the brain, C synthesis was increased (Fig. 4E–G), presumably to offset the deficit of C in the metabolic pool, because sterol was again being sequestered in the late E/L compartment. As a consequence of these changes, 6 weeks after administration of the CYCLO, the whole-animal cholesterol pool was still reduced by >900 mg/kg even though synthesis was increased by 48 mg·d<sup>-1</sup>·kg<sup>-1</sup> (Fig. 4D and H).

The residual effects of this single dose of CYCLO on the clinical measures of disease were even more dramatic. The liver function tests, aspartate aminotransferase and alanine aminotransferase, were markedly improved (Fig. 4I and J), and the level of neurodegeneration in the brain was significantly reduced. The *npc1*<sup>-/-</sup> mice had accumulations of vesicular storage material within the perikarya of neurons in multiple areas of the brain, most prominently in the neocortex, diencephalon, and ventral brainstem nuclei. In the cerebellum, there was marked loss of Purkinje cells, particularly in the anterior-superior cerebellar vermis, and this loss was accompanied by a proportional degree of Bergmann gliosis (Fig. 5A and B). Storage material identical to that seen in the other perikarya was present in the cytoplasm of the remaining Purkinje cells. This cell death was also strikingly expressed in loss of calbindin immunoreactivity in the molecular layer (Fig. 5D and E), reflecting loss of Purkinje cell dendrites, and by a marked increase in GFAP





**Fig. 3.** A single dose of CYCLO 4.5 was administered to 7-day-old  $npc1^{-/-}$  and  $npc1^{+/+}$  pups, and tissues were obtained 24 h later. RNA was extracted from the liver and brain for measurement by quantitative real-time PCR. (A) The mRNA level for ACAT2 is shown. The mRNA levels for hepatic target genes regulated by SREBP2 (B–E) and LXR (F–J) are shown in columns 1 and 2, respectively. (K–O) The third column provides relative mRNA levels in liver for various inflammatory proteins. (P–T) The fourth column of data shows several of these same relative mRNA values in the brain. Statistically significant differences are indicated by different letters ( $n = 6$ ,  $P < 0.05$ ).

immunoreactivity (Fig. 5 G and H), indicating activation of glial cells in the areas of Purkinje cell loss. This neurodegeneration occurred in a band-like distribution similar to that described (21). Although the single dose of CYCLO administered at 7 days of age did not alter the number of pyramidal cells present in the cerebral cortex of these 49-day-old animals (Fig. 4K), the number of surviving Purkinje cells in the cerebellum was more than doubled (Fig. 4L, 5C). This beneficial effect was also reflected in the substantial increase in calbindin immunoreactivity and the reduction in GFAP immunoreactivity seen in the cerebellum (Fig. 5 F and I), compared with untreated animals.

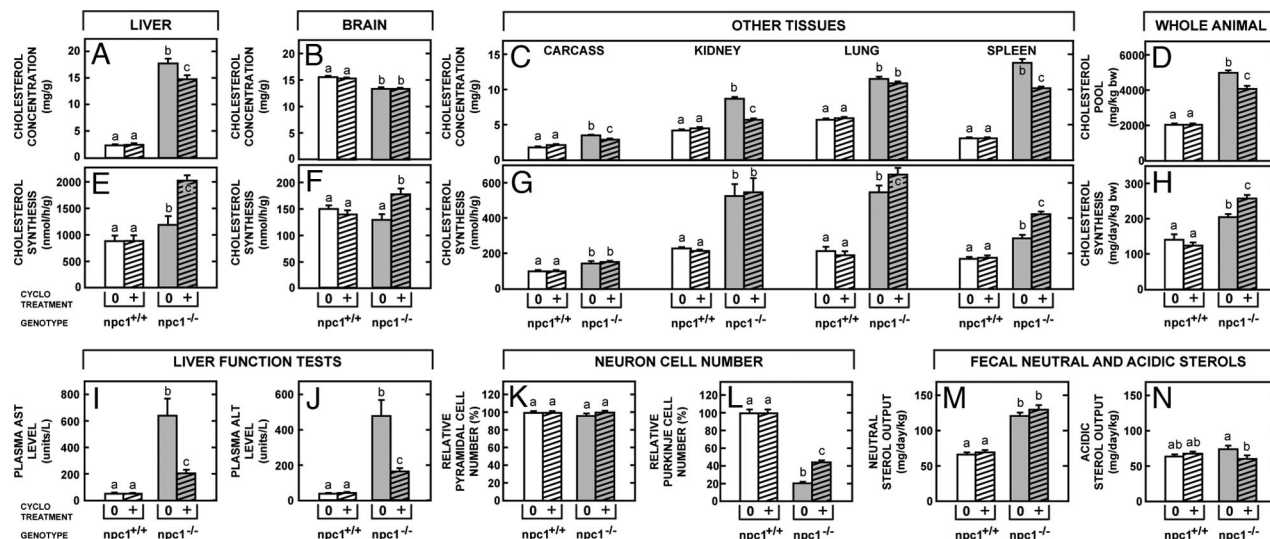
Finally, at 49 days of age, external sterol balance in the CYCLO-treated  $npc1^{-/-}$  mice had returned to levels seen in the untreated animals (Fig. 4 M and N). The sum of the fecal neutral and acidic sterol outputs equaled  $\approx 190 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$  in both groups, which was significantly higher than the  $120 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$  found in the untreated  $npc1^{+/+}$  animals. Again, CYCLO administration had no effect on sterol balance in these control mice. Thus, apparently, the single dose of cyclodextrin had markedly lowered the whole-animal pool of C in the days after its administration at 7 days of age and significantly slowed the development of liver and central nervous system disease. However, by 49 days of age, this effect had worn off; the animals were again sequestering sterol at a rate of  $\approx 70 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ , and external sterol balance had returned to the elevated levels usually found in  $npc1^{-/-}$  mice.

## Discussion

To variable degrees, nearly all cells in the body take up lipoproteins carrying apoE or apoB<sub>100</sub>. The CE present in the hydrophobic core of these particles is processed in lysosomes by a lysosomal acid lipase

(LAL) to C (22), which, in turn, is moved across the limiting membrane to the metabolically active pool of sterol in the cytosolic compartment by the concerted actions of NPC1 and NPC2. Mutations that inactivate LAL lead to accumulation of CE in the lysosomal compartment (Wolman disease) (23), whereas inactivation of NPC1 or NPC2 causes the sequestration of C (NPC disease). In the  $npc1^{-/-}$  mouse, although this mutation leads to expansion of the C pool in the late E/L compartment (4), there is a perceived shortage of sterol in the rest of the cell. As a result, there is marked activation of the SREBP2 target genes with an increase in sterol synthesis, no stimulation of the LXR target genes, and almost no CE formation (Figs. 2 and 3). In the liver, which expresses high levels of LDLR and where the reflection coefficient of the capillary bed is essentially zero, the rate of C sequestration equaled  $79 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$  during the 8 days between birth and when these animals were studied. The rate of accumulation in the remaining tissues of the body was  $87 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ .

After administration of CYCLO, there were rapid changes in the distribution of sterol within cells of all tissues. In the liver, for example, the rate of C movement out of the sequestered pool of sterol suddenly increased to  $233 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ , a rate that was 3-fold greater than the rate of sequestration. This shift of C into the metabolically active pool was reflected in profound suppression of SREBP2 and its target genes, leading to nearly complete inhibition of *de novo* sterol synthesis, activation of the LXR target genes ABCG1 and CYP7A1, and rapid expansion of the CE pool in the cytosol (Figs. 2 and 3). Of the 233 mg of C leaving the late E/L pool during this 24-h period, most moved to the ester pool in the cytosol ( $174 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ ), whereas the remainder must have been excreted from the liver ( $59 \text{ mg} \cdot \text{d}^{-1} \cdot \text{kg}^{-1}$ ) either as C or, after conversion, to



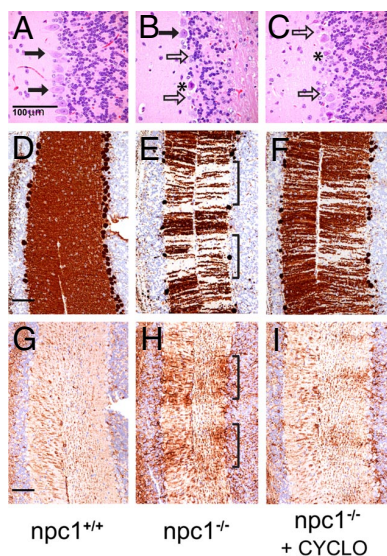
**Fig. 4.** *Npc1*<sup>+/+</sup> and *npc1*<sup>-/-</sup> mice were administered CYCLO 4.5 at 7 days of age and were then studied at 49 days of age. Total cholesterol concentrations were determined in liver (A), brain (B), and various other tissues (C), and these were combined to give whole-animal cholesterol pools (D). Similarly, cholesterol synthesis was measured in these same tissues (E–G), and these data were combined to give whole-animal synthesis rates (H). In these same groups of animals, liver function tests were also measured (I and J), pyramidal and Purkinje cell numbers were quantitated in the brain (K and L), and daily fecal neutral and acidic sterol output was determined (M and N). Statistically significant differences are indicated by different letters (n = 6, P < 0.05).

bile acid. In this manner, the hepatocytes attempted to adjust for the sudden influx of C into the metabolically active pool by immediately suppressing synthesis, sequestering much of the excess C as relatively inert CE, and excreting the remainder into bile. Similar,

although less dramatic, changes were identified in the brain and remaining tissues of the body (Fig. 2). As a result, within only 24 h of administration, CYCLO had reduced the total C pool in the 8-day-old pups by nearly 500 mg/kg. Presumably, this increase in net excretion continued for several more days until the CYCLO was fully excreted from the body.

CYCLO is a hydrophilic molecule containing a lipophilic pocket capable of solubilizing C and other hydrophobic molecules (24). It facilitates the movement of C between membranous structures by reducing the activation energy for desorption of the C molecule from  $\approx 20$  kcal/mol to 7 kcal/mol (25). Depending on the concentration and molar ratio of CYCLO to C, a solution of this molecule can either extract sterol from cell membranes (CYCLO/C molar ratio of  $>60$ ) or enrich the membrane with C (molar ratio  $<60$ ) (26). In addition, at low concentrations ( $<1$  mM), CYCLO can act catalytically, markedly enhancing the rate of molecular exchange of C between different hydrophobic compartments (27). Thus, one way in which CYCLO might have acted in the *npc1*<sup>-/-</sup> mice was to extract C from the plasma membranes of cells, facilitating its elimination from the body as the CYCLO was excreted by the kidneys. This is almost certainly not the case, however, in that there is no known pathway for C to move from the late E/L compartment directly to the plasma membrane in the *npc1*<sup>-/-</sup> cells. In addition, such a process would invariably lead to an increase in C synthesis and a reduction in the level of CE in cells. Importantly, no such effects were seen in the *npc1*<sup>+/+</sup> animals receiving CYCLO (Figs. 2 and 3), and in the *npc1*<sup>-/-</sup> mice, synthesis was actually suppressed, whereas the pool of CE was markedly expanded. A second possibility is that the CYCLO, reaching the late E/L compartment through bulk-phase endocytosis, altered the permeability of the limiting membrane in a nonspecific fashion, allowing C and other molecules to flow into the cytosolic compartment. This possibility also is unlikely, because any change in lysosomal permeability that released aspartic and serine proteases would likely lead to apoptosis and worsening of the disease seen in various organs (28). In the present studies, however, treatment with CYCLO actually decreased cell death and improved measures of tissue integrity (Figs. 4 and 5).

A third possibility is that CYCLO reaching the late E/L compartment through bulk-phase endocytosis either activated some, as



**Fig. 5.** Representative histological sections are shown of anterior-superior cerebellar vermis from 49-day-old *npc1*<sup>+/+</sup> (A, D, and G), *npc1*<sup>-/-</sup> (B, E, and H), and *npc1*<sup>-/-</sup> mice treated with CYCLO 4.5 at 7 days of age (C, F, and I). Contiguous sections were stained with H&E (A–C) or for calbindin (D–F) or GFAP (G–I) immunoreactivity. There was substantial loss of Purkinje cells (solid arrows) in untreated *npc1*<sup>-/-</sup> animals (B), which was partially prevented by treatment with CYCLO 4.5 (C). (B and C) Increased numbers of Bergmann glia (open arrows) and necrotic Purkinje cells (\*) were also visible in the untreated *npc1*<sup>-/-</sup> mice. (E) Calbindin immunoreactivity was markedly decreased in a band-like pattern in the molecular layer of untreated *npc1*<sup>-/-</sup> animals (brackets) reflecting loss of Purkinje cell dendrites. (H) In contrast, there was increased GFAP immunoreactivity in the molecular layer in the same regions of calbindin loss (brackets) reflecting activation of astrocytes. Treatment with CYCLO 4.5 markedly reduced this loss of calbindin expression (F) and diminished GFAP expression (I). (Measurement bars: 100  $\mu$ m.)



yet, undescribed secondary C transporter or, more likely, acted in a catalytic fashion to promote C transfer, bypassing the defective function of the mutant NPC1 protein. Originally, it seemed reasonable that during lysosomal processing of CE, the sequence of events was for LAL to release C to the soluble NPC2, which, in turn, would then transfer it to NPC1 for translocation across the limiting membrane. Recent studies, however, have raised the possibility that the direction of C movement may be from LAL to NPC1, from NPC1 to NPC2, and from NPC2 to some as yet undescribed transporter located in the limiting membrane (29, 30). If this scenario is correct, CYCLO might act in the late E/L compartment by shuttling C between LAL and NPC2, thereby bypassing the defective NPC1. This would fully account for the observation that the flow of C out of the late E/L compartment into the metabolically active pool suddenly increased to 233 mg·d<sup>-1</sup>·kg<sup>-1</sup>, leading to massive expansion of the CE pool, suppression of the SREBP2 system, and activation of certain LXR target genes.

These studies establish that the single dose of CYCLO administered at 7 days of age transiently overcame the transport defect in the late E/L compartment, temporarily suppressing further synthesis of sterol and allowing a major portion of the sequestered pool to be metabolized and excreted from the body. This reduced the total body burden of C by nearly 500 mg/kg after 24 h and by 900 mg/kg after 6 weeks. Even this transient effect markedly reduced the activation and influx of macrophages into the liver and brain, significantly improved liver function and Purkinje cell survival, and, ultimately, led to increased lifespan. The fact that significant alterations in brain biochemistry and nerve cell survival occurred after these injections clearly implied that the reflection coefficient for CYCLO in the central nervous system, although presumably high, was not 1.0. Given these very favorable biochemical and clinical alterations following the single injection, the question arises as to whether repeated injections of CYCLO over time might completely prevent the increase in total body C burden and, essentially, prevent the organ dysfunction seen in NPC disease.

Further, it must be established whether the reflection coefficient for CYCLO in the brain does become 1.0 as the animal ages and, therefore, whether such continuous CYCLO administration must be carried out both systemically, to prevent the lung and liver disease, and into the central nervous system, to halt the neurodegeneration.

## Materials and Methods

**Animals.** WT (*npc1*<sup>+/+</sup>) and homozygous mutant (*npc1*<sup>-/-</sup>) mice were generated from heterozygous (*npc1*<sup>+/-</sup>) animals with a pure BALB/c background (6, 15). Most pups were genotyped at 19 days of age, except in those experiments using 7-day-old animals, where the pups were genotyped at 5 days of age. All animals were housed in plastic colony cages in rooms with alternating 12-h periods of dark and light and were studied in the fed state. For the experiments using 49-day-old mice, there were comparable numbers of males and females in every group. The gender of the mice studied at 8 days of age was not determined. The dark phase began at 24:00 h and lasted 12 h. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical School.

**Treatments and Diets.** All animals except the heterozygous breeding stock were fed ad libitum a cereal-based low-cholesterol (0.02% wt/wt) diet (no. 7001; Harlan Teklad) or a ground form of this same diet containing ezetimibe (Schering-Plough Research Institute) (0.0125% wt/wt, providing ≈20 mg·d<sup>-1</sup>·kg<sup>-1</sup> bw) on weaning at 19 days of age. The breeding stock was maintained on another formulation containing 0.03% wt/wt cholesterol (no. 7002; Harlan Teklad). Mice were administered a single s.c. injection during the late dark phase at the scruff of the neck at 7 days of age of a 20% (wt/vol in saline) solution of CYCLO (4,000 mg/kg bw) with either 5.6° of substitution (product 332607; Aldrich) or 4.5° of substitution (product H107; Sigma). In some experiments, Allo (P 8887; Sigma) was added to the CYCLO solutions at a concentration of 1.5 mg/ml (25 mg/kg bw) (15, 16). Matching mice injected with saline only served as controls. All mice were studied either 24 h or 42 days later at the end of the dark cycle, when the animals were either 8 days or 49 days old, respectively.

**CYCLO Pharmacokinetics.** Whole-body CYCLO clearance was measured in 7- and 49-day-old mice using <sup>14</sup>C-CYCLO. Aliquots of the stock solution of <sup>14</sup>C-CYCLO (1.0 mCi/ml) were diluted with a 20% (wt/vol) solution of nonradiolabeled CYCLO just before administration to the mice, which were subsequently killed at various time points up to 24 h. The proportion of the administered <sup>14</sup>C-CYCLO dose that was retained by the body at each time point was determined by scintillation spectrometry after complete chemical digestion of the entire carcass. These data were used to calculate the proportion of the administered dose of <sup>14</sup>C-CYCLO that had been cleared from the animal over each respective time interval.

Additional materials and methods are described in [supporting information \(SI\) Materials and Methods and Table S1](#).

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## Cyclodextrins in the treatment of a mouse model of Niemann-Pick C disease

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### Abstract

Niemann-Pick type C (NPC) is a neurodegenerative disorder characterized by greatly altered somatic cholesterol metabolism. The *NPC1* gene has recently been cloned and shown to have sequence homology to other sterol-sensing proteins. We have used a mouse model with a disrupted *npc1* gene to study the effects of the cholesterol-mobilizing compound, 2-hydroxypropyl- $\beta$ -cyclodextrins (HPBCD), on the clinical course of this disorder. Treatment with two HPBCDs, with varying levels of 2-hydroxypropyl substitution, had effects in delaying neurological symptoms and in decreasing liver cholesterol storage while a third HPBCD was without effect. The ameliorating effect was not improved by longer exposure times (commencement of exposure *in utero*), however, it is not known if there is transplacental transfer of HPBCDs. The combination of HPBCD with probucol or nifedipine (which have previously been shown to lower liver cholesterol in this animal model) markedly decreased liver storage of unesterified cholesterol without altering the depressed levels of esterified cholesterol. The slight effects of the HPBCDs on neurological symptoms may be partially due to their apparent non-permeation of the blood-brain barrier (BBB). This non-permeation was assayed with radioactive tracers and was also present in the *mdr1a* knockout mice which have greatly increased BBB permeability for many drugs. Intrathecal delivery of HPBCD by an Alzet<sup>®</sup> osmotic minipump did not improve its efficacy in ameliorating neurological symptoms. © 2001 Elsevier Science Inc. All rights reserved.

**Keywords:** Cholesterol; Neurodegenerative disorders; Mouse models

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## Introduction

Niemann-Pick disease type C (NPC) is an autosomal recessive, neurodegenerative disorder of panethnic distribution. A major biochemical finding in NPC is the intracellular accumulation of unesterified cholesterol within lysosomes and the Golgi apparatus [1]. The *NPC1* gene has recently been cloned in man [2] and mice [3] and the predicted protein contains a sterol-sensing domain and other structural motifs. This suggests a direct causative role for the mutant *NPC1* product in altered cholesterol movement in NPC, although it is not known whether cholesterol accumulation is causative of the clinical features of NPC1 (there is a second locus, NPC2). For example, there has been no evidence of cholesterol storage in the brain [4]. The observation that the growth of NPC fibroblasts in lipoprotein-deficient media prevents cholesterol accumulation [5] suggests that further investigation is warranted before concluding that a reduction in cholesterol storage is ineffective.

Treatment of NPC patients with cholesterol-lowering agents has been shown to lower plasma and liver cholesterol levels, but included within this report was a 4-month follow up indicating no significant clinical effect [6]. Dimethyl sulfoxide has been claimed to elicit clinical improvement in one patient [7], while cholestyramine and lovastatin were reported to improve the storage of lipid as assessed by MRI after 3 months in another case [8]. We have previously studied the effects of probucol, nifedipine and the knockout of the low density lipoprotein (LDL) receptor in the mouse model of NPC [9]. These studies showed significant effects on liver cholesterol storage with little effect on neurologic outcome. We have now studied the effects of HPBCDs in this mouse model of NPC1. The HPBCDs are cyclic oligosaccharides consisting of seven  $\beta$ -(1–4)-glucopyranose units. The ring of sugar units creates a hydrophilic exterior (the hydroxypropyl substitution makes the  $\beta$ -cyclodextrins even more hydrophilic) with a hydrophobic interior that has been shown to have a very high affinity for sterols [10, 11, 12]. *In vitro* studies have shown very marked removal of cholesterol from a variety of cell lines [13] and from macrophage foam cells [14] by HPBCDs. These compounds lowered serum cholesterol levels when given parenterally in rats [15], and pigs fed HPBCDs and a cholesterol-enriched diet were largely protected from the hypercholesterolemic effects of the diet [16]. We observed that some parenterally-delivered HPBCDs lowered liver unesterified cholesterol levels and delayed neurological symptoms in the *npc* mouse despite their non-penetration of the BBB. Intrathecal delivery of a parentally efficacious HPBCD did not exhibit improved effects.

## Methods

### Mice

*npc1*<sup>−/−</sup> mutant mice on the BALB/cJ background were maintained by brother-sister mating of heterozygous animals. *mdr1a*<sup>−/−</sup> mice were the knockouts developed by Schinkel and colleagues [17] and were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed at the University of Arizona Animal Care Facility (PHS Assurance No. A-3248-01) on mouse chow containing 6% fat (or 10% for breeding mothers) and water *ad libitum*. At weaning (usually at 21–Mice, 28 days of age), tail tips were removed from mice and DNA

was prepared. Polymerase chain reactions (PCRs) to identify genotypes at the *npc1*—/— locus were performed using the primer pairs described in footnote 28 of Loftus and others [3]. The PCR conditions used 10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM Mg<sup>++</sup>; 200  $\mu$ M dNTPs, 1.25 U Taq polymerase, and 1  $\mu$ M of each primer. Twenty to 40 ng of DNA were added at 85°C, cycles of 30 sec. at 95°C, 30 sec. at 61°C, 1 min. at 72°C  $\times$  35, and 10 min. at 72°C were used. The products were separated on 1.2% NuSieve agarose gels.

### Chemicals

2-hydroxypropyl- $\beta$ -cyclodextrin (HPBCD) of average TDS 4.2 was obtained from Sigma Chemical Co. (Lot Number 86H0822); HPBCD of TDS 4.3 (Encapsin, HPB, Lot Number 05L-101/1) was obtained from Janssen Biotech, NV; Olen Belgium; and HPBCD of TDS 6.7 (Lot Number G8120) was obtained from Cerestar, Inc., Hammond, IN. These TDS values may not reflect the original literature/catalog information which frequently is in error due to confusion about TDS nomenclature [18]. These 3 different formulations of HPBCD were dissolved in sterile-normal saline at a concentration such that they could each be delivered in 0.01 ml per gram body weight.

Probucol (a gift from Dr. Gordon A. Francis and Dr. Shinji Yokohama, The University of Alberta) was dissolved in Captex 300 (Abitec Corporation, Janesville, WI). Captex 300 is a medium-chain triglyceride vehicle for delivery of probucol that does not alter serum cholesterol levels when given alone [19]. The 200 mg/kg (given 2 or 3 times/week in separate series) was chosen since it is an effective dose in rats [19] and was previously effective in lowering liver cholesterol in mice [9].

Nifedipine (Sigma, St. Louis, MO) was initially dissolved in 100% ethanol that was mixed further with Captex 300 to achieve a final concentration of 20% ethanol, 80% Captex 300. The HPBCD dosage of 0.87 mg/kg/day is about one-fourth the LD<sub>50</sub> (Merck Index, 1976) and its concentration was adjusted such that it could be delivered in a volume of 0.01 cc per gram body weight. It was also previously effective in lowering liver cholesterol in mice [9].

<sup>14</sup>C-labeled HPBCD (specific activity 6.95 mCi/g) of TDS of 5.15 was obtained from Cyclodextrin Technologies Development, Inc. (Gainesville, FL). [Fructose-1-<sup>3</sup>H(N)]-sucrose with a specific activity of 12.3 Ci/mmol was obtained from Sigma Chemical Co. (St. Louis, MO). The bradykinin agonist, RMP-7, was kindly provided by Alkermes, Inc. (Cambridge, MA).

### Drug delivery

Drugs were delivered by intraperitoneal injection using a track under the skin before the peritoneum was entered to prevent efflux of the agent. In one case, a pregnant female was continuously placed on HPBCD with a total degrees of substitution (TDS) of 6.7 (see below) at 500 mg/kg, given 3 times/week intraperitoneally (we do not know if the HPBCD can cross the placenta). This animal delivered a series of litters (there seemed to be no effect on the *npc1* +/— mouse's fertility or viability at this dose) and the pups were tail-tipped at about 14 days. Thus, if any of the HPBCD was crossing into the breast milk, the pups were receiving it during lactation as well. At weaning, *npc1*—/— mice were started on the same dose of HPBCD intraperitoneally. Eventually, 3 affected homozygous mice were studied in this way.

### *Cholesterol determinations*

Cholesterol concentrations were determined using the cholesterol oxidase method of Heider and Boyett [20]. Briefly, the liver homogenates were extracted with hexane:isopropanol (3:2) for 1 hour, followed by centrifugation to pelletize the protein. The organic solution was removed, dried and spotted onto a thin-layer chromatography plate (Redi-Plate, Silica Gel G, Fisher Scientific, Tustin, CA). To separate neutral lipids, hexane:ethyl ether:glacial acetic acid (80:30:1.1) was used as the mobile phase. After staining with iodine vapors, unesterified cholesterol and esterified cholesterol were scraped from the plate and hydrolyzed using 1.00 ml of 1.0 N KOH in absolute ethanol. Cholesterol was then extracted with 3 ml of 100% hexane, and an aliquot dried in a glass tube. To each tube, 20  $\mu$ l isopropanol (cholesterol assay grade) and 0.4 ml of assay reagent containing 0.1 ml cholesterol oxidase (8 U/ml), 0.1 ml peroxidase (3000 U/ml), 1.0 ml of *p*-hydroxyphenylacetic acid (0.15 mg/ml) and 8.8 ml of 0.05 M sodium phosphate buffer (pH 7.0) was added and incubated for 20 min at room temperature. The reaction was terminated by adding 0.8 ml of 0.5 N NaOH. A Perkin-Elmer Model LS-40 fluorescence spectrometer (excitation at 325 nm and emission at 415 nm) was used for measuring fluorescence. Unknown cholesterol concentrations were determined by reference to known cholesterol standards using linear regression, and were normalized to the amount of protein determined using a BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL).

### *Blood-brain barrier permeability studies*

Normal BALB/c, *npc1*<sup>−/−</sup> and *Mdr1a*<sup>−/−</sup> (the knockout of the multiple drug resistance P-glycoprotein; [17]) mice with body weights between 17 and 24 g were used. Chemicals for intravenous injections were formulated such that 10  $\mu$ l of radiotracer solution per g of body weight was injected into the tail vein of mice. One ml of injection solution contained 5  $\mu$ Ci of <sup>14</sup>C-hydroxypropyl- $\beta$ -cyclodextrin and 25  $\mu$ Ci of <sup>3</sup>H-sucrose diluted in sterile PBS containing 50 mg of unlabeled HPBCD, TDS 4.2, 1% (w/v) unlabeled sucrose, and 0.1% bovine serum albumin. For the RMP-7 experiments, only normal BALB/c animals were used. RMP-7 was delivered concurrently with the tracers, such that a working concentration of 10  $\mu$ g of RMP-7 per kg of body weight was attained. Animals were sacrificed by cervical dislocation 60 minutes after intravenous bolus administration. The brain was quickly removed from the skull, weighed and homogenized in PBS. Three hundred  $\mu$ l aliquots of homogenized tissue were transferred to 10 ml of counting fluid (Scintiverse II; Fisher Scientific, Tustin, CA), and total radioactivity was determined by double-isotope liquid scintillation counting in a Beckman LS 5000 TD counter (average counting efficiencies of 76% for <sup>14</sup>C and 46% for <sup>3</sup>H). All samples were assayed in duplicate.

### *Intrathecal delivery of 2-hydroxypropyl- $\beta$ -cyclodextrin*

The intraventricular stereotaxic coordinate was determined to be 2 mm posterior to the bregma, 1 mm from the midline, and at a 4 mm depth from the cranium. BALB/c *npc1*<sup>−/−</sup> mice, of approximately 30 days old, were weighed and anesthetized with Avertin [21]. Osmotic minipumps (Alzet Model 2004, Palo Alto, CA) were filled with HPBCD of 4.2 TDS (20 mg/ml in 0.9 % sodium chloride solution). Cannulae from Alzet's 3–5 mm Brain Infusion Kit were used with 2 spacers leaving a cannula length of 4 mm. The catheter tubes were cut to a

size 25 percent longer than the distance between the subcutaneous site of the pump and the location of the cannula. The scalp was shaved and cleaned and a 2 cm midline incision was made through the scalp to the cranium using small scissors. The cranium was exposed by pulling the skin aside with stay sutures and the periosteal connective tissue was scraped off using a scalpel. A subcutaneous pocket was prepared in the midscapular area using a hemostat. A hole was drilled just through the cranium at the stereotaxic site using an 18G needle. Next, the osmotic pump was inserted into the subcutaneous pocket so that the cannula lined up with the hole. Dental cement (Missy Inc., Cherry Hill, NJ) was prepared and generously applied to the bottom spacer on the cannula taking care to avoid setting cement on the tip of the cannula. The cannula was inserted into the brain perpendicular to the cranium and held in place until the cement set. More cement was prepared and applied around the cannula and cranium and allowed to set. The incision was closed using 4-0 silk sutures (Ethicon, Somerville, NJ). The animal was returned to its cage for recovery. The pumps continuously delivered the HPBCD at a rate of 0.25  $\mu$ l/hour for 28 days. This procedure was performed multiple times on mice of similar age and weight and was followed by a very slow infusion of methylene blue to confirm the intraventricular location.

### *Evaluations and statistical analyses*

Onset of neurological symptoms was defined as the time when a continuous extensor tremor of the forelimbs occurred for more than 6–7 seconds when the animal was picked up by the tail. Mice were observed 3 times a week for this variable. By the time such extensor tremor developed, the animals had moderately severe ataxia. We have found that daily evaluations of the extent and duration of the forelimb tremor were more reproducible than was the degree of ataxia. We initially also determined the maximal weight (averaged between the 2 sexes as there is a sexual dimorphism in weight) gained as another indication of health status. *npc1*–/– homozygous mice grow until they are unable to access food and water well because of their neurological disorder, at which point they start losing weight. We have calculated the difference between the average maximum male weight and the average maximum female weight (since there are major sexual dimorphisms in the weight of mice), divided by 2. We then subtracted this weight from the maximum male weight and added it to the maximum female weight to get a mid-sex weight for quantitative comparisons. Student's t-test was used to compare these variables, including the esterified and unesterified cholesterol. Results are presented in Tables 1 and 2 as means  $\pm$  1 standard deviation.

## **Results**

### *Effects of HPBCD of TDS 4.2*

As seen in Table 1, the 100 mg/kg dose, given 3 times/week, decreased unesterified cholesterol by approximately 50% and increased esterified cholesterol. The dose of 500 mg/kg, (average start 28 days) given 3 times/week, had even more marked effects on lowering unesterified cholesterol but had somewhat less of an effect on increasing esterified cholesterol. When we introduced the start of the 500 mg/kg dose earlier (i.e., 23 days), also given 3 times/week, it did not have a particularly greater effect on unesterified cholesterol but, in this case,

Table 1

Treatment with 2-hydroxypropyl- $\beta$ -cyclodextrin (average total degree of substitution 4.2) alone and in combination with probucol and nifedipine

Treatment	N	Mean age at onset of continuous symptoms or death (days)	Mean unesterified cholesterol ( $\mu\text{g}/\text{mg}$ protein)	Mean esterified cholesterol ( $\mu\text{g}/\text{mg}$ protein)
None, normals	10		$7.8 \pm 0.8^+$	$3.1 \pm 0.1^+$
None, <i>Npc-1</i>	10	$50.3 \pm 3.2^+$	$153.3 \pm 35.^+$	$0.7 \pm 0.1^+$
HPBCD				
100 mg/kg, 3/wk	5	$52.4 \pm 3.0$	$79.2 \pm 10.7^{**}$	$2.2 \pm 0.4^{****}$
HPBCD				
500 mg/kg, 3/wk (avg. start 28 days)	7	$59.6 \pm 4.7^{**}$	$8.6 \pm 16.7^{**}$	$1.3 \pm 0.6^*$
HPBCD				
500 mg/kg, 3/wk (avg. start 23 days)	4	$61.5 \pm 4.5^{****}$	$49.0 \pm 22.5^{****}$	$7.1 \pm 2.7^{****}$
HPBCD				
500 mg/kg, probucol 200 mg/kg, 3/wk	3	$56.3 \pm 2.1$	$25.0 \pm 5.2^{****}$	$0.50 \pm 0.2$
HPBCD				
250 mg/kg, nifedipine 0.87 mg/kg, 1/day	3	$48.7 \pm 3.5$	$22.0 \pm 1.5^{***}$	$0.7 \pm 0.1$

<sup>+</sup> from [9].

\*  $p < 0.005$ ; \*\*  $p < 0.001$ ; \*\*\*  $p < 0.0005$ ; \*\*\*\*  $p < 0.0001$ .

mean esterified cholesterol was markedly increased to more than twice normal levels. The cyclodextrin treatments initiated at either an average of 23 days or 28 days significantly delayed the time of onset of continuous symptoms to about 60 days, i.e., a roughly 20% extension of the “non-symptomatic” period (by the criteria chosen). Interestingly, the combination of 200 mg/kg of probucol with 500 mg/kg cyclodextrin, both given 3 times/week, resulted in very marked decreases in unesterified cholesterol but was without effect on esterified cholesterol. A similar result occurred when HPBCD was combined with nifedipine. Again, very marked decreases in unesterified cholesterol were observed but little effect on normalizing esterified cholesterol or time of onset of symptoms was seen.

#### *Effects of HPBCD with other TDS and in utero commencement of treatment*

As seen in Table 2, HPBCD of TDS 4.3 did not cause any change in the timing of neurological symptoms. We repeated the experiments with the original HPBCD of TDS 4.2 several more times, not recording mean male/female maximum weight, since statistically significant effects were not found for this variable, and not repeating the determinations of unesterified or esterified cholesterol. The results were as before with a significant prolongation of the symptom-free period. HPBCD of TDS 6.7 also significantly delayed the onset of neurologi-

Table 2

Treatment with 2-hydroxypropyl- $\beta$ -cyclodextrin of various total degrees of substitution

Treatment	N	Mean age at onset of continuous symptoms	Mean unesterified cholesterol (ug/mg protein)	Mean esterified cholesterol (ug/mg protein)
None, normals	10		$7.8 \pm 0.8^+$	$3.1 \pm 0.3^+$
None, <i>Npc-1</i>	10	$50.3 \pm 3.2^+$	$153.35 \pm 35.40$	$0.68 \pm 0.13$
4.2 TDS <sup>++</sup>				
500 mg/kg; 3/wk	4	$63.2 \pm 3.****$	n.r. <sup>++</sup>	n.r.
4.2 TDS <sup>++</sup>				
500 mg/kg; 3/wk started at weaning	4	$62.2 \pm 2.6****$	n.r.	n.r.
6.7 TDS				
500 mg/kg; 3/wk to mother before birth and through lactation, then to weaning	3	$53.0 \pm 10.7$	(2) <sup>++</sup> $45.1 \pm 3.2$	$1.7 \pm 0.1***$
4.3 TDS				
500 mg/kg; 3/wk	9	$51.6 \pm 6.3$	(3) $10.2 \pm 3.8****$	(3) $4.4 \pm 1.3****$
6.7 TDS				
500 mg/kg; 3/wk	9	$57.5 \pm 8.4^*$	(3) $27.8 \pm 4.5****$	$5.9 \pm 2.6****$

<sup>+</sup> from [9]<sup>++</sup> separate experiments from data in Table 1; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.005$ .\*\*\*\*  $p < 0.001$ .<sup>++</sup> not repeated, see Table 1.

cal symptoms, but there was a great deal of variability and the effects seemed not as marked as that found with the HPBCD TDS 4.2. Continuous presentation of the HPBCD of TDS 6.7, starting *in utero*, actually was less efficacious (but trans-placental and trans-lactation transfer is not known) and the results were not different from untreated animals for the duration of symptoms, although unesterified cholesterol was significantly lowered and esterified cholesterol elevated.

#### Blood-brain barrier permeability studies

We studied the permeability of the BBB for HPBCDs using a single 1 hour time point and radiolabeled HPBCD of TDS 5.15 (the only radiolabeled HPBCD available). Tritium labeled sucrose was used as the control for “dead volume”, i.e., blood volume not washed out in the brain. As seen in Figure 1, HPBCD precisely paralleled the distribution of sucrose in these mice. The amount of HPBCD and sucrose in normal mice (about 2.5% of the injected dose in 1 hour, the differences in molarity represent the different amounts needed for counting due to the difference in specific activities) should represent retained blood. Homozygosity for the *npc1* mutation results in a two-fold enhanced dead volume ( $p < 0.01$  for HPBCD,  $p < 0.005$  for sucrose) which may represent brain shrinkage in the degenerating *npc1*–/– brain. The *mdr1a* knockout did not significantly increase the BBB permeability/vascular volume for



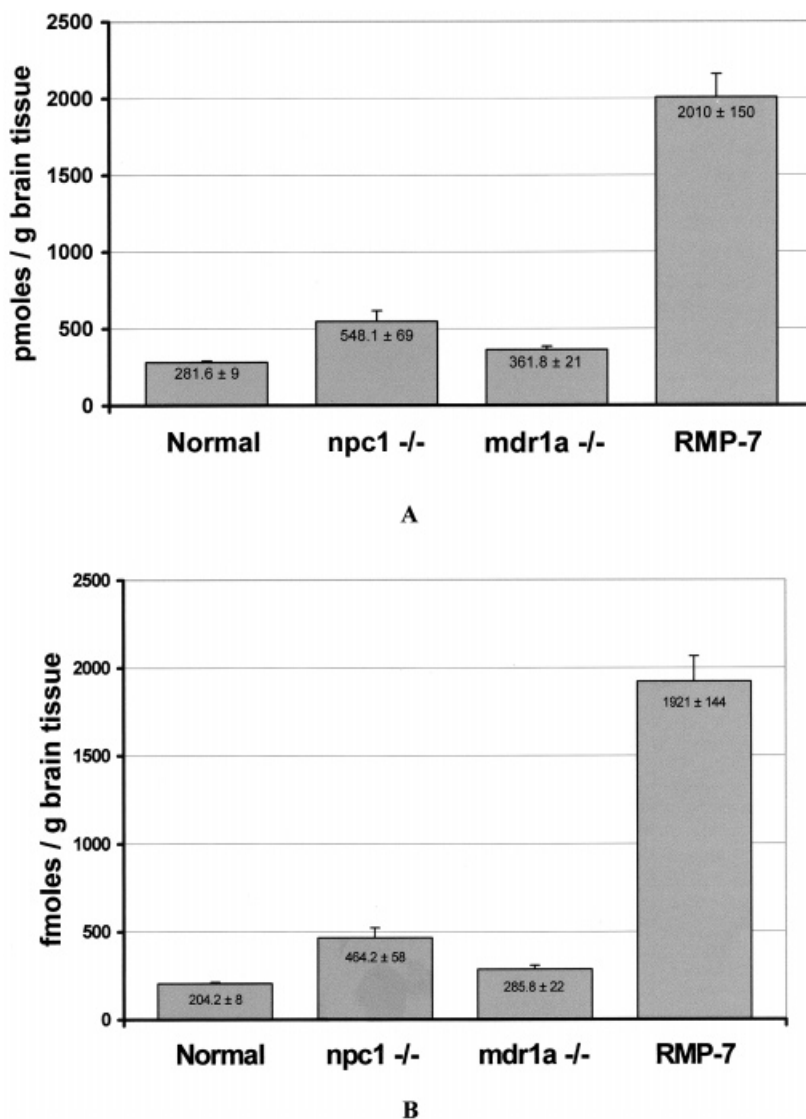


Fig. 1. Brain content of tracer at 1 hour. Brain delivery was measured as moles of tracer per g of brain parenchyma (a) [<sup>14</sup>C]hydroxypropyl-beta-cyclodextrin or (b) [<sup>3</sup>H] sucrose.

HPBCD or sucrose. In our final experiments, the bradykinin agonist, RMP-7, was used to acutely make the BBB permeable. This was very effective in allowing the penetration of both HPBCD and sucrose ( $p < 0.007$  for HPBCD,  $p < 0.0002$  for sucrose).

#### *Intrathecal administration of HPBCD of 4.2 TDS*

Inasmuch as the HPBCD was not significantly penetrating the BBB, we tried intrathecal administration of the drug. Mice with the cannula and osmotic minipump were very active

and frequently broke the cemented cannula away from the skull. Three mice were successfully infused until neurological symptoms were continuous. The average age of onset of continuous symptoms was  $47 \pm 4.6$  days, not statistically different from untreated controls.

## Discussion

Our studies in mice [9] and some studies in humans [6] did not show permanent beneficial effects of several cholesterol-lowering agents on neurological symptoms in NPC1. HPBCDs have proven to be powerful reagents for studying cholesterol metabolism in cultured cells [13, 14] and have allowed the demonstration that the mobilization of cholesterol from lysosomes to the endoplasmic reticulum is mostly mediated via the plasma membrane [22]. Although the parent  $\alpha$ - and  $\beta$ -cyclodextrins cause renal toxicity, such damage is not observed for the hydroxypropyl derivatives such as HPBCD [23].

We have used parenterally-administered HPBCDs with different TDS alone, and in combination with, other cholesterol-lowering drugs in the *npc1* null mice. Although there was some variation in the time at which treatment was started, HPBCDs of TDS 4.2 and 6.7 delayed the onset of neurological symptoms by about 20%. On the other hand, an HPBCD of TDS 4.3 did not alter the course of the neurological disease. All of the HPBCDs lowered unesterified cholesterol but exhibited more variable effects on elevating esterified cholesterol. The most marked effects on lowering the storage of unesterified cholesterol in the liver were found with a combination of cyclodextrins and either probucol or nifedipine; drugs previously shown to lower liver cholesterol in *npc1*<sup>-/-</sup> mice [9]. However, esterified cholesterol was not elevated when combinations of drugs were used. The drug combinations that had a greater effect on lowering liver unesterified cholesterol did not significantly delay neurological symptoms but only small numbers were studied.

We believe that the difference between the two neurologically effective and one non-effective HPBCD must lie in other variables. For example, two HPBCD samples with the same “average degree of substitution” may not have identical physicochemical properties since the hydroxypropyl groups may occupy different positions on the parent molecule [18].

It was of interest that there was an increased “dead volume”/permeability in the *npc1*<sup>-/-</sup> mice compared to their congenic normal controls, the BALB/cJ strain. This may reflect shrinkage of the brain or a real increase in BBB permeability. We have previously described an upregulation of caveolin-1 in *npc1*<sup>-/-</sup> mice, although it was not nearly as great as the upregulation which occurred in the *npc1*<sup>+/-</sup> mice [24]. Caveolin uptake of drugs has been implicated in their movement across cells [25], and it is possible that this is the mechanism involved. Alternatively, the many changes in cholesterol homeostasis which occur in NPC1, provide many potential levels in which the BBB might be altered. It was of interest that the *mdr1a*<sup>-/-</sup> mice did not show an increased permeability of the BBB for HPBCDs. The *mdr1a* P-glycoprotein is well known for its role in causing multidrug resistance of tumors [26]. The *mdr1a* knockout has shown increased BBB permeability for a wide variety of pharmaceutical agents [27]. Perhaps, the failure of the *mdr1a* knockout to enhance the permeability is related to the large size and hydrophilicity of the HPBCDs. Thus, since sucrose uptake was not enhanced by the mutation, it is not surprising that the HPBCD uptake was not enhanced. One study of the clearance of cyclodextrins delivered intracerebrally suggest that the efflux was independent of the BBB [28].

We also used the bradykinin agonist, RMP-7, to study altered permeability of the BBB. RMP-7 relaxes the tight junctional complexes of the BBB without inducing damage to microvessels [29]. The increased permeability of the BBB is very short lasting with restoration of normal permeability occurring within minutes for several pharmaceutical agents [30]. The short time duration can be overcome with continuous intravenous infusions of RMP-7 [31]. For our chronic neurologic disease, a continuous infusion would not be practical, but it was of interest that RMP-7 significantly enhanced the BBB permeability of HPBCD. Thus, an approach to altering tight junctional complexes of endothelial cells, rather than manipulation of the *mdr1a* pathway, might be the appropriate approach for increasing central nervous system delivery of HPBCDs. However, since the delay of symptoms from parental cyclodextrins was small and intrathecal delivery of somatically efficacious HPBCD did not delay the onset of neurological symptoms, these currently available compounds are unlikely to be helpful in patients.

## Conclusion

HPBCDs had quite marked effects on somatic cholesterol metabolism with some small effects on the central nervous system in *npc1*<sup>−/−</sup> mice. The results obtained here, although preliminary in nature, indicate that the HPBCDs are worthy of further study in the treatment of NPC1 disease.

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# Chronic Cyclodextrin Treatment of Murine Niemann-Pick C Disease Ameliorates Neuronal Cholesterol and Glycosphingolipid Storage and Disease Progression

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## Abstract

**Background:** Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disorder caused most commonly by a defect in the NPC1 protein and characterized by widespread intracellular accumulation of unesterified cholesterol and glycosphingolipids (GSLs). While current treatment therapies are limited, a few drugs tested in *Npc1*<sup>-/-</sup> mice have shown partial benefit. During a combination treatment trial using two such compounds, *N*-butyldeoxymojirimycin (NB-DNJ) and allopregnanolone, we noted increased lifespan for *Npc1*<sup>-/-</sup> mice receiving only 2-hydroxypropyl- $\beta$ -cyclodextrin (CD), the vehicle for allopregnanolone. This finding suggested that administration of CD alone, but with greater frequency, might provide additional benefit.

**Methodology/Principal Findings:** Administration of CD to *Npc1*<sup>-/-</sup> mice beginning at either P7 or P21 and continuing every other day delayed clinical onset, reduced intraneuronal cholesterol and GSL storage as well as free sphingosine accumulation, reduced markers of neurodegeneration, and led to longer survival than any previous treatment regime. We reasoned that other lysosomal diseases characterized by cholesterol and GSL accumulation, including NPC disease due to NPC2 deficiency, GM1 gangliosidosis and mucopolysaccharidosis (MPS) type IIIA, might likewise benefit from CD treatment. Treated *Npc2*<sup>-/-</sup> mice showed benefits similar to NPC1 disease, however, mice with GM1 gangliosidosis or MPS IIIA failed to show reduction in storage.

**Conclusions/Significance:** Treatment with CD delayed clinical disease onset, reduced intraneuronal storage and secondary markers of neurodegeneration, and significantly increased lifespan of both *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice. In contrast, CD failed to ameliorate cholesterol or glycosphingolipid storage in GM1 gangliosidosis and MPS IIIA disease. Understanding the mechanism(s) by which CD leads to reduced neuronal storage may provide important new opportunities for treatment of NPC and related neurodegenerative diseases characterized by cholesterol dyshomeostasis.

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**Competing Interests:** SUW is an author on a patent for the use of miglustat for the treatment of Niemann-Pick C disease. MTV has been a paid and non-paid consultant for Actavis, which markets miglustat as treatment for Niemann-Pick C disease.

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## Introduction

Niemann-Pick type C (NPC) disease is an autosomal recessive neurodegenerative disorder characterized by accumulation of unesterified cholesterol and glycosphingolipids (GSLs), such as GM2 and GM3 ganglioside [1,2]. Patients with this fatal disease develop an ataxic gait and motor dysfunction, typically preceded by vertical gaze palsy and organomegaly, and later accompanied by seizures and dementia. On a cellular level, neurons exhibit complex disease-related morphological alterations including formation of meganeurites, ectopic dendrites, and axonal spheroids [3]. NPC disease is also characterized by neurodegeneration, including the presence of intracellular protein aggregates in the form of

neurofibrillary tangles, and a well-defined patterned loss of cerebellar Purkinje cells [4]. Defects in a transmembrane protein, NPC1, account for approximately 95% of cases with the remainder involving a soluble protein, NPC2 [2]. Both proteins have been shown to bind cholesterol [5–8] and are found in the late endosomal/lysosomal (LE/LY) system. Here they are thought to function cooperatively in facilitating egress of cholesterol from LE/LY to other sites in the cell [9,10]. Cholesterol sequestration in NPC disease is generally believed to be followed secondarily by GSL accumulation, but restricting synthesis of complex gangliosides has been shown to reduce cholesterol storage in most neurons [11,12].

Therapeutic options for NPC disease are quite limited. Substrate reduction therapy (SRT) utilizes drugs that reduce the



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## Abstract

**Background:** Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disorder caused most commonly by a defect in the NPC1 protein and characterized by widespread intracellular accumulation of unesterified cholesterol and glycosphingolipids (GSLs). While current treatment therapies are limited, a few drugs tested in *Npc1*<sup>-/-</sup> mice have shown partial benefit. During a combination treatment trial using two such compounds, *N*-butyldeoxynojirimycin (NB-DNJ) and allopregnanolone, we noted increased lifespan for *Npc1*<sup>-/-</sup> mice receiving only 2-hydroxypropyl- $\beta$ -cyclodextrin (CD), the vehicle for allopregnanolone. This finding suggested that administration of CD alone, but with greater frequency, might provide additional benefit.

**Methodology/Principal Findings:** Administration of CD to *Npc1*<sup>-/-</sup> mice beginning at either P7 or P21 and continuing every other day delayed clinical onset, reduced intraneuronal cholesterol and GSL storage as well as free sphingosine accumulation, reduced markers of neurodegeneration, and led to longer survival than any previous treatment regime. We reasoned that other lysosomal diseases characterized by cholesterol and GSL accumulation, including NPC disease due to NPC2 deficiency, GM1 gangliosidosis and mucopolysaccharidosis (MPS) type IIIA, might likewise benefit from CD treatment. Treated *Npc2*<sup>-/-</sup> mice showed benefits similar to NPC1 disease, however, mice with GM1 gangliosidosis or MPS IIIA failed to show reduction in storage.

**Conclusions/Significance:** Treatment with CD delayed clinical disease onset, reduced intraneuronal storage and secondary markers of neurodegeneration, and significantly increased lifespan of both *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice. In contrast, CD failed to ameliorate cholesterol or glycosphingolipid storage in GM1 gangliosidosis and MPS IIIA disease. Understanding the mechanism(s) by which CD leads to reduced neuronal storage may provide important new opportunities for treatment of NPC and related neurodegenerative diseases characterized by cholesterol dyshomeostasis.

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**Competing Interests:** SUW is an author on a patent for the use of miglustat for the treatment of Niemann-Pick C disease. MTV has been a paid and non-paid consultant for Actelion, which markets miglustat as treatment for Niemann-Pick C disease.

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## Introduction

Niemann-Pick type C (NPC) disease is an autosomal recessive neurodegenerative disorder characterized by accumulation of unesterified cholesterol and glycosphingolipids (GSLs), such as GM2 and GM3 ganglioside [1,2]. Patients with this fatal disease develop an ataxic gait and motor dysfunction, typically preceded by vertical gaze palsy and organomegaly, and later accompanied by seizures and dementia. On a cellular level, neurons exhibit complex disease-related morphological alterations including formation of meganeurites, ectopic dendrites, and axonal spheroids [3]. NPC disease is also characterized by neurodegeneration, including the presence of intracellular protein aggregates in the form of

neurofibrillary tangles, and a well-defined patterned loss of cerebellar Purkinje cells [4]. Defects in a transmembrane protein, NPC1, account for approximately 95% of cases with the remainder involving a soluble protein, NPC2 [2]. Both proteins have been shown to bind cholesterol [5–8] and are found in the late endosomal/lysosomal (LE/LY) system. Here they are thought to function cooperatively in facilitating egress of cholesterol from LE/LY to other sites in the cell [9,10]. Cholesterol sequestration in NPC disease is generally believed to be followed secondarily by GSL accumulation, but restricting synthesis of complex gangliosides has been shown to reduce cholesterol storage in most neurons [11,12].

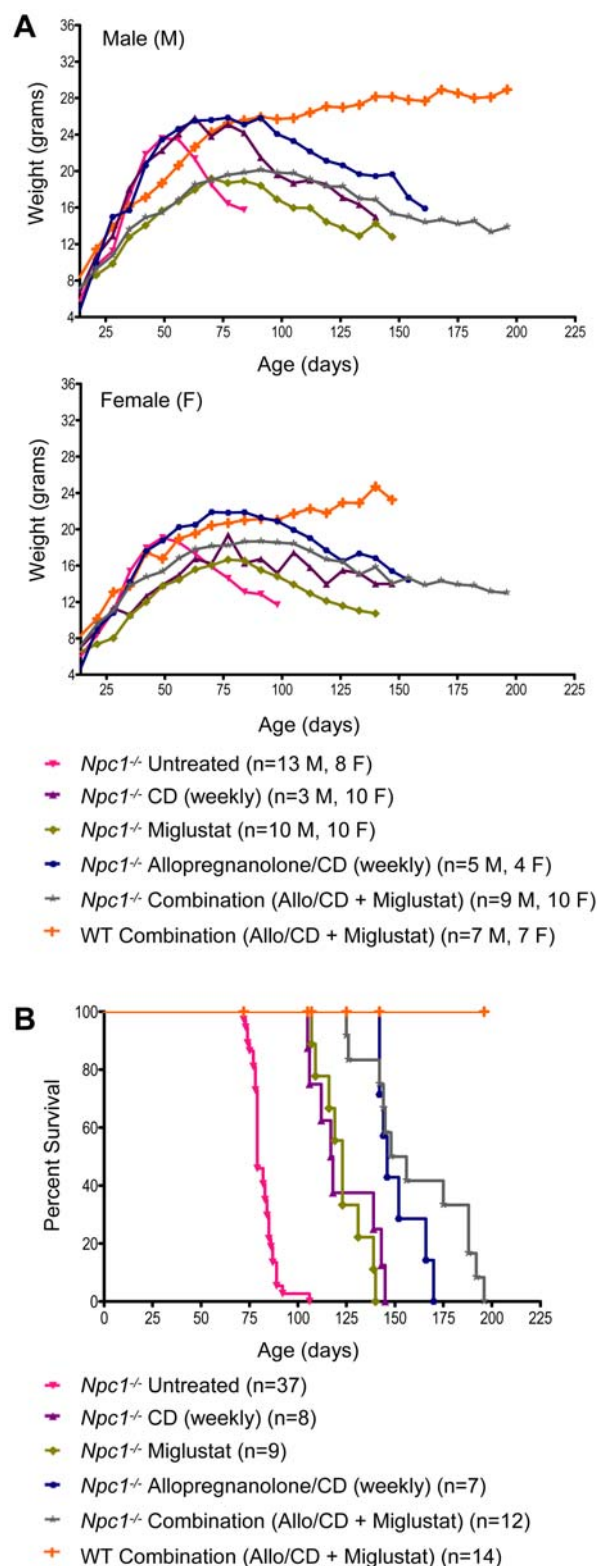
Therapeutic options for NPC disease are quite limited. Substrate reduction therapy (SRT) utilizes drugs that reduce the

synthesis of metabolic precursors or products which themselves are known to accumulate in storage diseases [13]. In 2001, Zervas and colleagues demonstrated that daily administration of an inhibitor of GSL synthesis, *N*-butyldeoxynojirimycin (NB-DNJ) or miglustat to *Npc1*<sup>-/-</sup> mice resulted in a reduction in accumulation of GSLs, a delay in onset of clinical signs, and a 30% increase in lifespan [14]. More recently, miglustat has also been shown in a randomized controlled study to stabilize or improve clinical markers of NPC disease in patients treated for 12 months [15] and in January of 2009, miglustat (Zavesca®) was approved by the European Medicines Agency for use in treatment of neurological symptoms in patients with NPC disease. A second therapeutic agent tested in *Npc1*<sup>-/-</sup> mice was allopregnanolone, a neurosteroid shown deficient in the central nervous system (CNS) of *Npc1*<sup>-/-</sup> mice [16]. Administration of allopregnanolone solubilized in 2-hydroxypropyl- $\beta$ -cyclodextrin (CD) to *Npc1*<sup>-/-</sup> mice at postnatal day 7 (P7) was reported to be beneficial, with treated mice exhibiting delayed clinical onset, extended life span, and reduced ganglioside accumulation. This therapeutic approach has been referred to as by-product replacement therapy or BRT, since the strategy was thought to replace missing or deficient products needed for normal cellular functioning [17]. Based on these positive results, we reasoned that a combination therapy utilizing both miglustat and allopregnanolone might work synergistically to ameliorate NPC1 disease. While this combination therapy did prove additive, our studies revealed that the vehicle, CD, also provided significant benefit. This finding coincided with published observations that single dose CD increased the lifespan of *Npc1*<sup>-/-</sup> mice (18). Additional studies reported here further establish that CD treatment alone dramatically ameliorates NPC disease and questions the efficacy of allopregnanolone administered without CD. A recent publication investigating the effects of a single injection of CD with or without allopregnanolone in NPC1 disease reported no additional benefit of allopregnanolone in reducing cholesterol accumulation in the liver and brain as well as a reduction in markers of neurodegeneration, but did reconfirm the increase in lifespan of CD-treated *Npc1*<sup>-/-</sup> mice. [19]. Our current report further demonstrates that sequestration of GSLs, sphingosine, and cholesterol is significantly reduced in neurons of CD-treated *Npc1*<sup>-/-</sup> mice and that chronic treatment with CD leads to the most significant amelioration of NPC disease in the murine model seen to date.

## Results

### Combination SRT and BRT therapy in *Npc1*<sup>-/-</sup> mice provides synergistic effect

*Npc1*<sup>-/-</sup> mice treated with a combination therapy of NB-DNJ (administered daily starting at P10) and allopregnanolone/CD (administered weekly starting at P7) showed a 2 week delay in onset of clinical signs (ataxic gait, tremor) when compared to untreated (given no injections) or vehicle (saline or CD) treated *Npc1*<sup>-/-</sup> mice. Untreated *Npc1*<sup>-/-</sup> mice began a precipitous weight loss beginning at 6 weeks of age, while combination-treated *Npc1*<sup>-/-</sup> mice gradually lost weight starting at 14 weeks (Fig. 1A). Combination-treated *Npc1*<sup>-/-</sup> mice lived significantly longer than did untreated or vehicle-injected (saline or CD) *Npc1*<sup>-/-</sup> mice (Fig. 1B). We noted, however, that *Npc1*<sup>-/-</sup> mice receiving CD alone also lived significantly longer than untreated *Npc1*<sup>-/-</sup> mice (median age: untreated *Npc1*<sup>-/-</sup> mice: 79 days; CD-treated *Npc1*<sup>-/-</sup> mice: 118 days;  $p < 0.0001$ ). Correspondingly, weight loss in CD-injected *Npc1*<sup>-/-</sup> mice was delayed by approximately 3 weeks. Analysis of cholesterol accumulation by filipin labeling revealed a decrease in cholesterol storage in neocortical neurons of combination and CD-treated *Npc1*<sup>-/-</sup> mice compared to



**Figure 1. Combination treatment using NB-DNJ and allopregnanolone/CD in *Npc1*<sup>-/-</sup> mice.** (A) Average weight over time for each treatment group shown for males and females separately. (B) Survival of each treatment group. Median survival of *Npc1*<sup>-/-</sup> mice: no treatment, 79 days; CD (weekly), 118 days; Miglustat (daily), 123 days; Allopregnanolone/CD (weekly), 146 days; Combination therapy (Miglustat + Allopregnanolone/CD), 152 days. doi:10.1371/journal.pone.0006951.g001

untreated *Npc1*<sup>-/-</sup> mice (Fig. 2A). Like cholesterol, GM2 and GM3 gangliosides are well characterized storage components of NPC disease, but their levels in wild-type (WT) brain are negligible. Immunohistochemical (IHC) analysis of GM2 and GM3 gangliosides in the neocortex of *Npc1*<sup>-/-</sup> mice yielded similar results in terms of cholesterol storage, with combination and CD-treated mice exhibiting less ganglioside accumulation than untreated mice (Fig. 2B, C). Comparison of cerebellum in age-matched untreated and combination-treated *Npc1*<sup>-/-</sup> mice revealed a striking rescue of Purkinje cells in treated animals as evidenced by calbindin labeling (Fig. 2D). While surviving Purkinje cells were confined largely to lobule X in untreated *Npc1*<sup>-/-</sup> mice, those receiving the combination drug treatment still had Purkinje cells present in all lobules. GM2 accumulation was present mainly in the granular cell layer of *Npc1*<sup>-/-</sup> mice, but combination-treated *Npc1*<sup>-/-</sup> animals had less GM2 staining than *Npc1*<sup>-/-</sup> controls (untreated and saline; data not shown).

### Short-term administration of CD alone reduces storage in *Npc1*<sup>-/-</sup> mice

The discovery that CD alone (without allopregnanolone) reduced intraneuronal storage and increased longevity of *Npc1*<sup>-/-</sup> mice led us to perform a series of studies to address its possible role as a therapeutic agent in and of itself. *Npc1*<sup>-/-</sup> mice were treated beginning at P7 with injections of CD every other day for 2 weeks. Route of CD administration was either subcutaneous (SC) or intraperitoneal (IP) and while both had a similar outcome, SC administration seemed to be slightly more efficacious (Fig. S1). At 2 weeks of age, mice were terminated to evaluate cholesterol and GSL storage. Results showed little to no accumulation of cholesterol in neurons of the cerebral cortex in 22-day old treated *Npc1*<sup>-/-</sup> mice, whereas age-matched untreated *Npc1*<sup>-/-</sup> mice exhibited considerable filipin-labeling (Fig. 3A). CD treatment also diminished accumulation of gangliosides, such that GM2 and GM3 storage in the neocortex of treated *Npc1*<sup>-/-</sup> mice was nearly absent compared to readily detectable storage in untreated *Npc1*<sup>-/-</sup> mice (Fig. 3B; GM3 not shown). A parallel quantitative biochemical study of gangliosides in cerebrum of these mice revealed strikingly different patterns in the untreated and treated *Npc1*<sup>-/-</sup> animals, with a near normalization to WT levels in the concentrations of both GM2 and GM3 after CD treatment (Fig. 3C). At this age, GM2 has already reached its near maximal level in the untreated mutants, while GM3 is at about 50% of its maximum (unpublished data, MT Vanier). Ultrastructural analysis revealed that cortical neurons of a 22-day old CD-treated *Npc1*<sup>-/-</sup> mouse had little to no evidence of polymembranous cytoplasmic bodies (PCBs) characteristic of NPC disease (Fig. 3D), a finding consistent with the lack of detectable cholesterol and ganglioside storage.

Analysis of cerebellar cortex from 22-day old untreated *Npc1*<sup>-/-</sup> mice revealed that nearly every Purkinje cell, as well as presumptive neurons in both the granule and molecular cell layers, showed evidence of cholesterol accumulation by filipin labeling (Fig. 3E). However, most Purkinje cells in age-matched CD-treated *Npc1*<sup>-/-</sup> mice lacked cholesterol storage and only a small number of filipin-positive neurons were present in the granule cell layer. IHC analysis of GM2 in the cerebellum of untreated *Npc1*<sup>-/-</sup> mice revealed prominent accumulation throughout the granule cell layer and occasional storage in the molecular cell layer, while age-matched CD-treated *Npc1*<sup>-/-</sup> mice exhibited less accumulation in both the granule and molecular cell layers (data not shown). Levels of the autophagosome marker LC3-II were also affected by CD treatment, as evidenced by western blot analysis of the cerebellum. Increased levels of LC3-II have previously been shown in the CNS of *Npc1*<sup>-/-</sup> mice suggesting alterations in the degradative mechanism known as

macroautophagy [20,21]. Our results confirmed this increase in LC3-II and additionally, showed that levels in CD-treated *Npc1*<sup>-/-</sup> mice were normalized to those seen in WT controls (Fig. 3F).

These findings indicating that CD significantly limits cholesterol and ganglioside storage in neurons of young *Npc1*<sup>-/-</sup> mice led us to carry out two additional studies, one examining the ability of allopregnanolone alone to ameliorate disease progression and the other to determine the efficacy of long-term CD therapy.

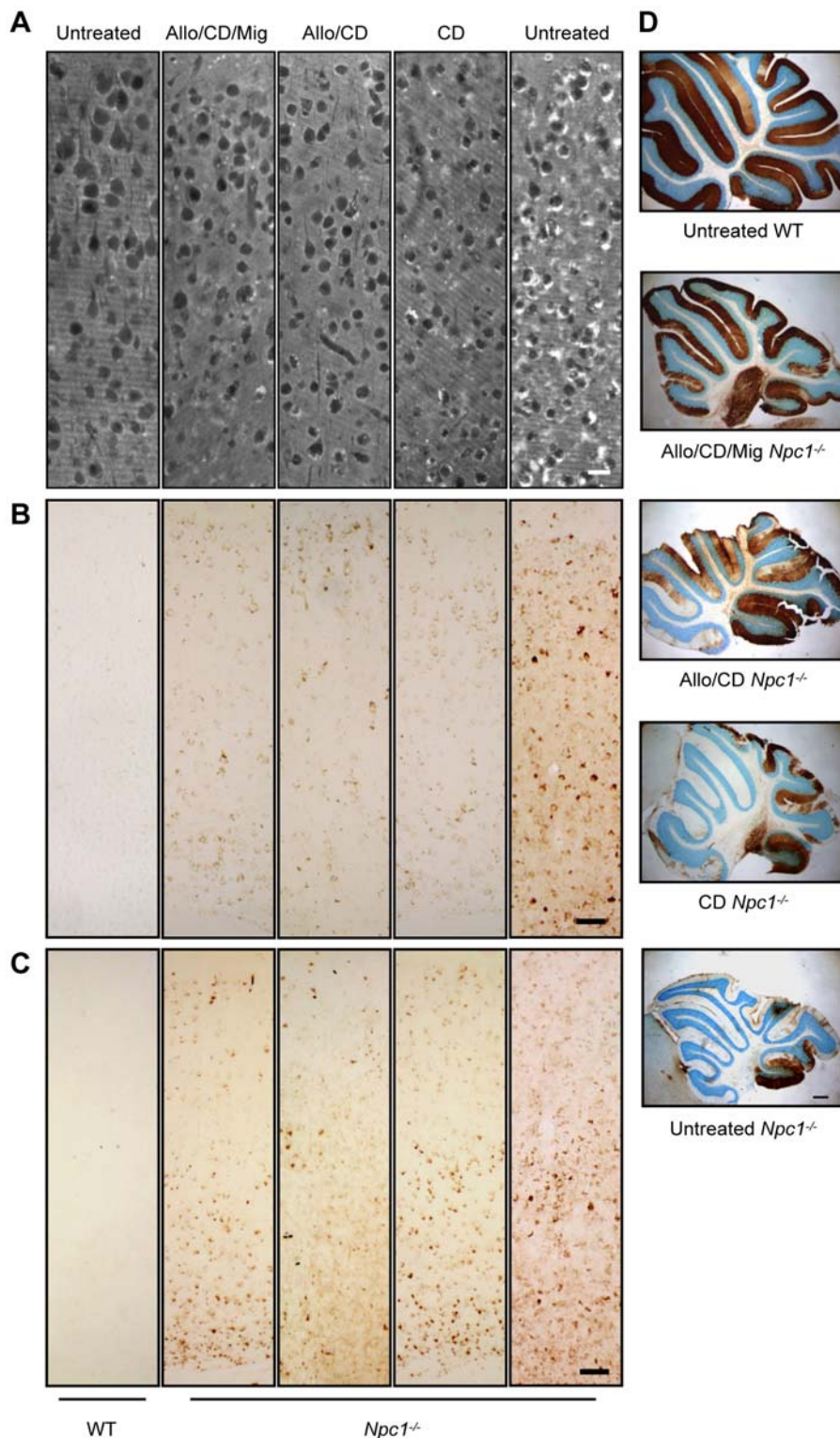
### Allopregnanolone without CD does not appear beneficial

The initial combination study suggested not only a beneficial effect of CD, but also a possible small additional benefit of allopregnanolone with CD (Fig. 1A; median age: CD-treated *Npc1*<sup>-/-</sup> mice: 118 days; Allopregnanolone/CD-treated *Npc1*<sup>-/-</sup> mice: 146 days;  $p < 0.0038$ ). To determine whether there was any beneficial impact of allopregnanolone alone on *Npc1*<sup>-/-</sup> mice, we administered allopregnanolone using vehicles other than CD (dimethyl sulfoxide [DMSO] or corn oil) or a reduced concentration of CD (5% CD solution). Mice were given weekly injections following the same protocol used in the combination study, with the exception of allopregnanolone/corn oil (total of three injections given at P7, P14, and P21). Onset of ataxic gait occurred in all treated and untreated *Npc1*<sup>-/-</sup> mice at 6–7 weeks of age and there was no increase in lifespan of the allopregnanolone-treated *Npc1*<sup>-/-</sup> mice (vehicle: corn oil or DMSO) compared to vehicle injected *Npc1*<sup>-/-</sup> controls. *Npc1*<sup>-/-</sup> mice treated with allopregnanolone solubilized in 5% CD did live significantly longer than mice receiving the other allopregnanolone solutions or corn oil and DMSO vehicles, but so too did the control animals receiving only 5% CD (Fig. 4A). Analysis of cholesterol and gangliosides did not demonstrate detectable storage reductions between the allopregnanolone-treated *Npc1*<sup>-/-</sup> mice versus their respective vehicle-injected controls. Collectively, these results suggest that the beneficial effects observed in the combination trial for allopregnanolone-treated *Npc1*<sup>-/-</sup> mice are due largely to the vehicle, CD, and that allopregnanolone may provide only a small additional benefit when administered in 20% CD.

### Chronic CD injections ameliorate NPC1 disease in mice

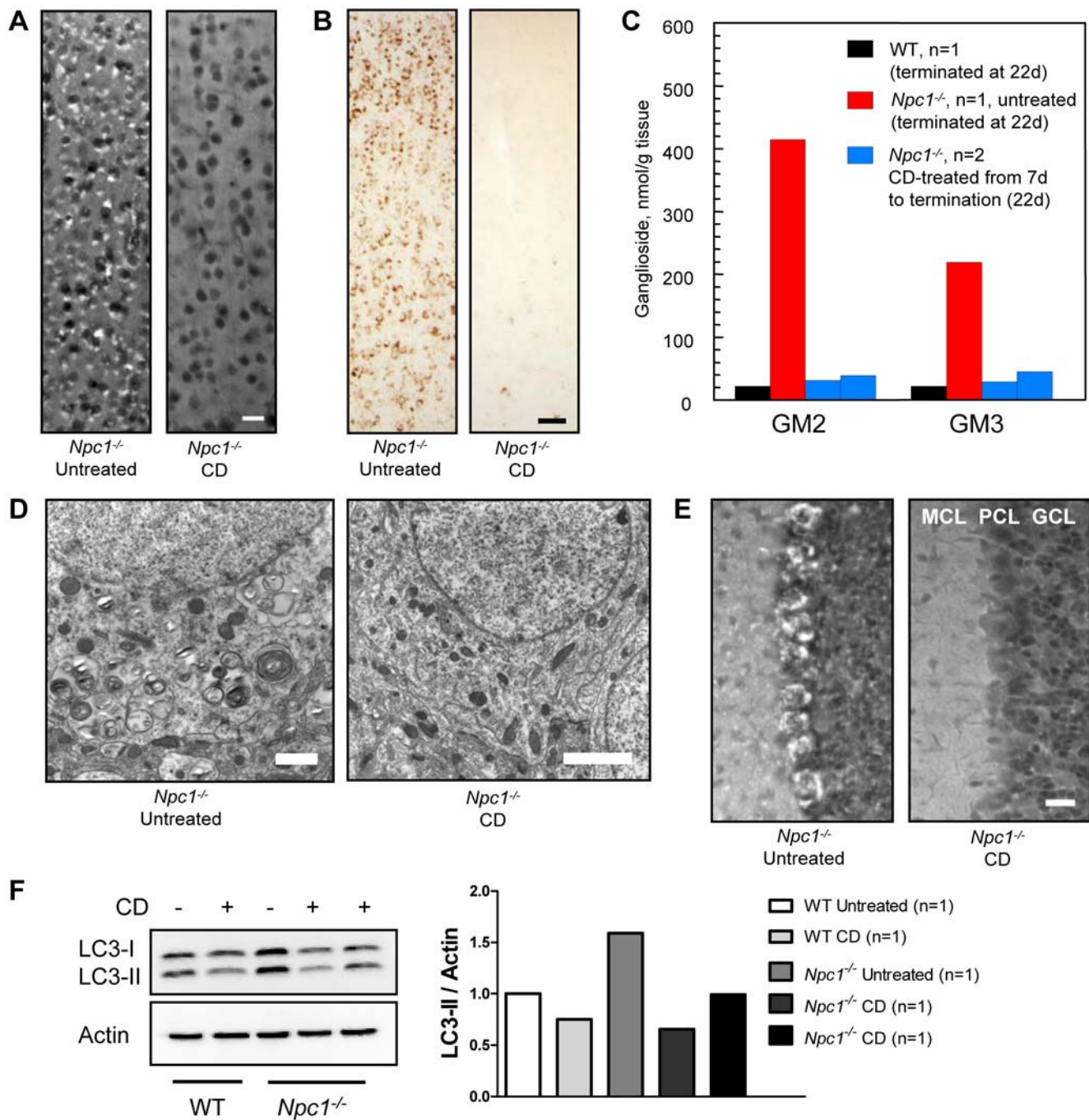
Using the same treatment regimen described earlier (injections given every other day), *Npc1*<sup>-/-</sup> and WT mice were administered CD beginning at P7 and continuing to end-stage disease, when affected mice showed a hunched posture, severe gait disturbance, and/or weight loss greater than 30% of peak weight. Behaviorally, onset of ataxia in untreated *Npc1*<sup>-/-</sup> mice occurred between 6 and 7 weeks of age, while CD treatment delayed onset by 3 weeks, such that treated *Npc1*<sup>-/-</sup> mice became ataxic between 9 and 10 weeks of age. Furthermore, abrupt weight loss in untreated *Npc1*<sup>-/-</sup> mice began to occur around 7 weeks of age, while treated *Npc1*<sup>-/-</sup> mice exhibited a gradual decline in weight beginning at 10 to 13 weeks of age (Fig. 4B). Every-other-day treated *Npc1*<sup>-/-</sup> mice on average lived longer than those on the original combination therapy, though the difference was not significant (Fig. 1B and 4C; median age: combination-treated *Npc1*<sup>-/-</sup> mice: 152 days; CD-treated *Npc1*<sup>-/-</sup> mice began at P7: 185 days;  $p < 0.2138$ ).

Filipin labeling of neocortical neurons in CD-treated *Npc1*<sup>-/-</sup> mice at end-stage revealed less cholesterol storage compared to their untreated counterparts, in spite of their significant age differences (Fig. 5A). There was a reduction in both the number of neurons that showed cholesterol storage and the amount of cholesterol in individual neurons, some of which appeared to have little or no accumulation (Fig. 5B). Confocal analysis of treated *Npc1*<sup>-/-</sup> mouse brain showed that many cholesterol negative



**Figure 2. Cholesterol and ganglioside immunohistochemistry (IHC) of *Npc1*<sup>-/-</sup> and WT mice in the combination treatment study.** (A) Filipin labeling of unesterified cholesterol (seen as white areas in image) in the neocortex of age-matched untreated and treated *Npc1*<sup>-/-</sup> and WT mice (all mice between 75 and 81 days of age) revealed less cholesterol accumulation of treated *Npc1*<sup>-/-</sup> mice (second, third, and fourth panels) when compared to control *Npc1*<sup>-/-</sup> mice (fifth panel). WT mice do not exhibit cholesterol accumulation (first panel). Each panel here and in (B) and (C) shows layers II (top) through VI (bottom) of the cerebral cortex. (B) IHC of GM2 ganglioside (visualized as brown punctae within cells) was also characterized by reduced GM2 storage in all treated *Npc1*<sup>-/-</sup> mice. (C) IHC of GM3 ganglioside (again seen as brown punctae within cells) showed results similar to GM2. (D) Treated *Npc1*<sup>-/-</sup> mice had more remaining Purkinje cells (brown areas in cerebellar images) than did untreated *Npc1*<sup>-/-</sup> mice; however, treated mice still had Purkinje cell loss when compared to WT mice. Anti-calbindin antibody labels Purkinje cell bodies and dendritic arbors, while the Nissl counterstain (purple) labels all neuronal cell bodies. Images taken at 20X (A), 10X (B, C), and 2X (D); scale bars 20  $\mu$ m (A), 50  $\mu$ m (B, C), and 400  $\mu$ m (D). doi:10.1371/journal.pone.0006951.g002



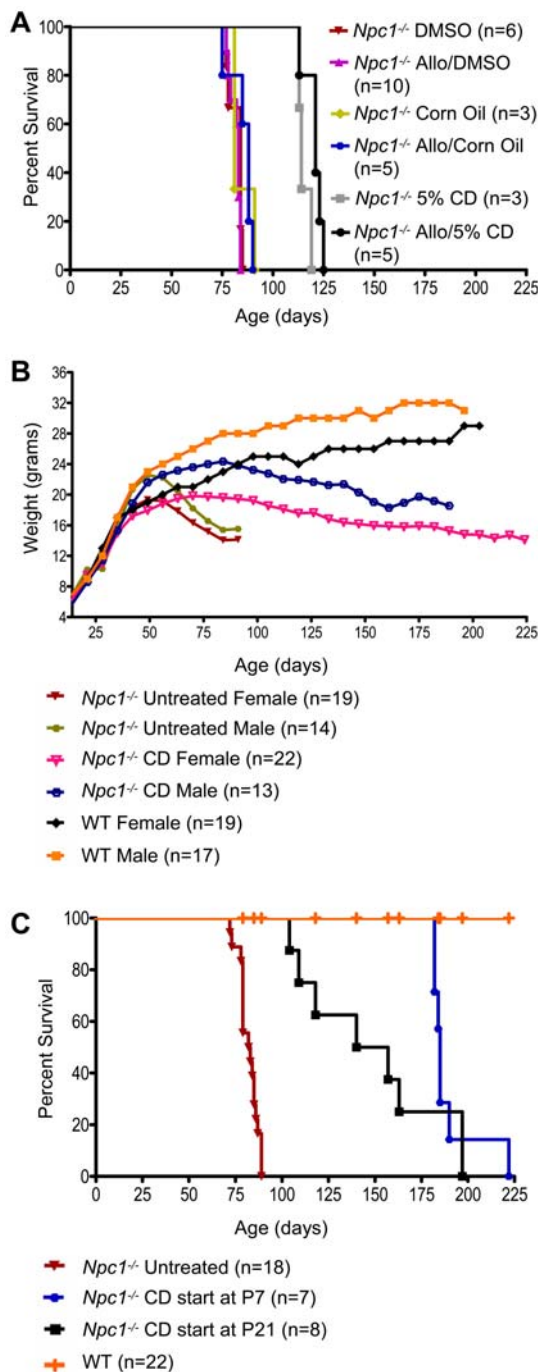


**Figure 3. Short term (2 week) CD study in *Npc1*<sup>-/-</sup> mice.** (A) Filipin labeling of unesterified cholesterol in the neocortex of untreated and CD-treated *Npc1*<sup>-/-</sup> mice revealed dramatically less cholesterol accumulation in CD-treated mice at 22 days of age. Mice were administered SC injections of CD every other day for 2 weeks starting at P7. (B) IHC of untreated and CD-treated *Npc1*<sup>-/-</sup> mice also revealed less GM2 storage present in CD-treated mice (similar finding for GM3, not shown). (C) Biochemical analysis of ganglioside levels further corroborated the reduction in GM2 and GM3 seen with IHC analysis. (D) Ultrastructural analysis of neocortical neurons in *Npc1*<sup>-/-</sup> untreated and CD-treated mice showed remarkably normal neuronal morphology in CD-treated mice. (E) Filipin labeling of unesterified cholesterol in the cerebellum of untreated and CD-treated *Npc1*<sup>-/-</sup> mice indicated little to no cholesterol accumulation present within Purkinje cells of CD-treated mice; cerebellar layers: molecular cell layer (MCL), Purkinje cell layer (PCL), and granular cell layer (GCL). (F) Western blot analysis of LC3-II, an autophagosome marker, revealed less LC3-II present in CD-treated *Npc1*<sup>-/-</sup> as compared to untreated *Npc1*<sup>-/-</sup> mice. Images taken at 20X (A, E) and 10X (B); scale bars 20  $\mu$ m (A, E), 50  $\mu$ m (B), 1  $\mu$ m (D). doi:10.1371/journal.pone.0006951.g003

neocortical neurons still exhibited ganglioside accumulation (Fig. 5C).

IHC staining of both GM2 and GM3 gangliosides revealed less accumulation in CD-treated *Npc1*<sup>-/-</sup> mice compared to age-matched *Npc1*<sup>-/-</sup> controls (Fig. 5D, GM3 not shown). Biochem-

ical analysis of gangliosides (Fig. 5E) corroborated and extended this finding. In the cerebrum of CD-treated *Npc1*<sup>-/-</sup> mice, the increase in GM2 and GM3 levels, while not significant at 22 days (Fig. 3C), remained very moderate at 90 days (115 and 76 nmol/g for GM2 and GM3, respectively, compared with 474 $\pm$ 19 and



**Figure 4. Chronic allopregnanolone and chronic CD treatment studies in *Npc1*<sup>-/-</sup> mice.** (A) Survival of untreated and allopregnanolone-treated *Npc1*<sup>-/-</sup> mice using different vehicles for allopregnanolone. Median survival of *Npc1*<sup>-/-</sup> mice: DMSO, 84 days; Allo/DMSO, 83 days; Corn Oil, 81 days; Allo/Corn Oil, 88 days; 5% CD, 114 days; Allo/5% CD, 121 days. (B) Average weight over time for untreated and CD-treated *Npc1*<sup>-/-</sup> and WT mice. Weights of untreated and CD-treated WT mice were averaged for each gender as there was no significant difference between treatments ( $p < 0.8125$ ). (C) Survival of untreated and CD-treated *Npc1*<sup>-/-</sup> and WT mice showing effects of different start times. Median survival of *Npc1*<sup>-/-</sup> mice: no treatment, 83 days; CD (every other day, start at P7), 185 days; CD (every other day, start at P21), 149 days. Treatment initiated at P7 appeared more efficacious, although lifespan was not significantly longer when compared to treatment initiated at P21 ( $p < 0.1870$ ). doi:10.1371/journal.pone.0006951.g004

429 $\pm$ 27 nmol/g in age-matched, end-stage untreated *Npc1*<sup>-/-</sup> mice; mean  $\pm$  SD). The two treated mice at end-stage (190 and 223 days) still showed concentrations of GM2 and GM3 gangliosides approximately half of those in end-stage untreated *Npc1*<sup>-/-</sup> mice. Neuro-inflammation is also an important component of many lysosomal diseases [22], and upon IHC staining of CD68, a CNS inflammatory marker, we noted reduced labeling in brains of end-stage CD-treated *Npc1*<sup>-/-</sup> mice compared to end-stage untreated *Npc1*<sup>-/-</sup> mice (data not shown). Ultrastructurally, although neurons of CD-treated *Npc1*<sup>-/-</sup> mice often had PCBs, they appeared to be less abundant than in untreated *Npc1*<sup>-/-</sup> mice (Fig. 5F).

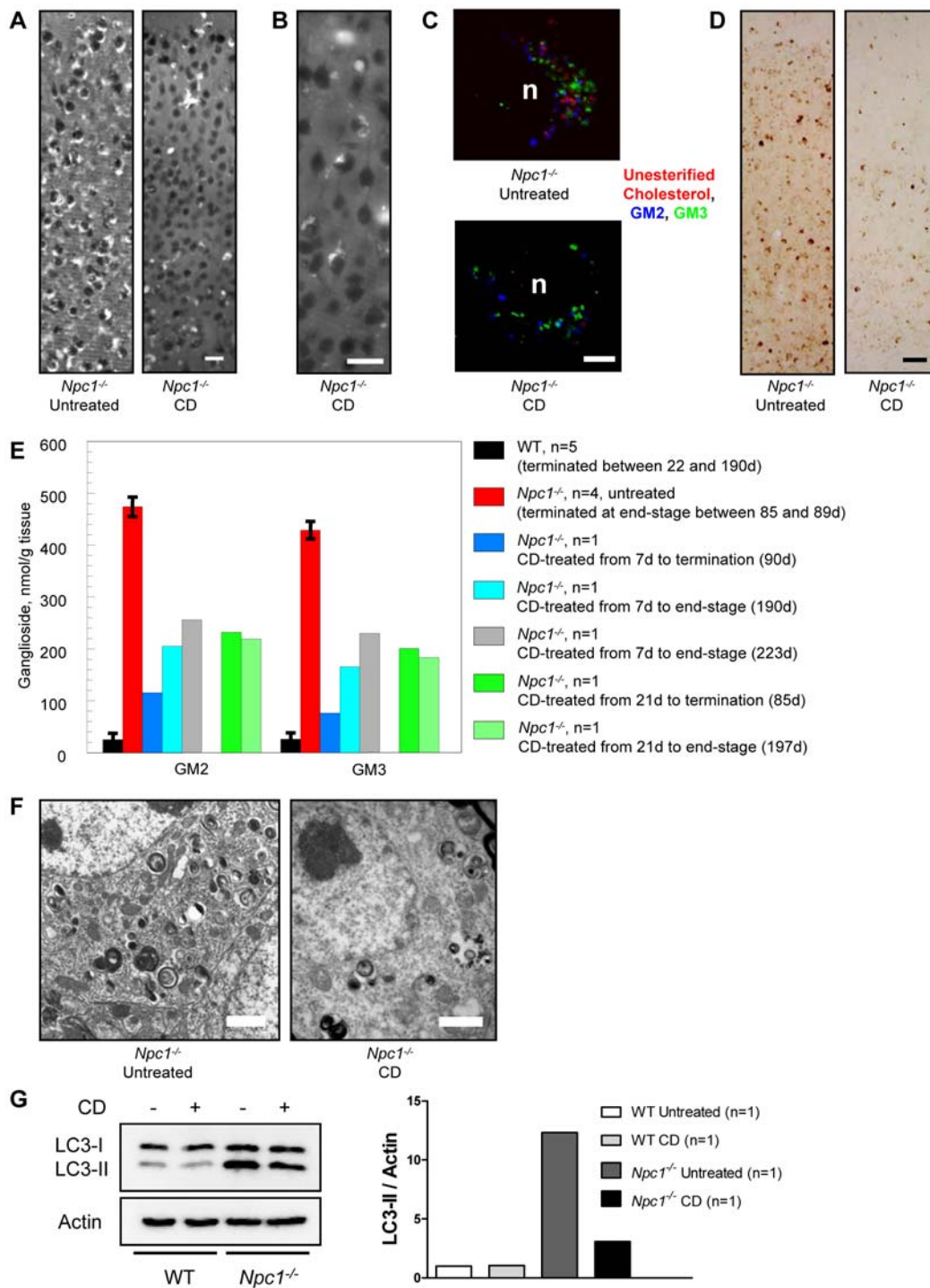
Cholesterol storage was observed in all Purkinje cells of the cerebellum in treated and untreated *Npc1*<sup>-/-</sup> mice. At end-stage, remaining Purkinje cells were observed almost exclusively in lobule X, whereas in treated *Npc1*<sup>-/-</sup> mice (age-matched to end-stage untreated *Npc1*<sup>-/-</sup> mice), rescued Purkinje cells were routinely observed in other lobules. IHC analysis of GM2 ganglioside revealed prominent accumulation throughout the granule cell layer and occasional storage within the molecular cell layer of end-stage untreated *Npc1*<sup>-/-</sup> mice cerebella. CD-treated *Npc1*<sup>-/-</sup> mice exhibited this same pattern but with reduced GM2 accumulation (data not shown).

As in the short term study, biochemical analysis of LC3-II in the cerebellum revealed that CD-treated *Npc1*<sup>-/-</sup> mice had levels similar to WT, while untreated *Npc1*<sup>-/-</sup> mice showed an increase in LC3-II levels compared to WT (Fig. 5G).

In addition to brain analyses, we examined peripheral tissues including kidney, liver, and lung for changes induced by treatment with CD. Filipin analysis of kidneys from the chronic CD treatment study revealed no obvious differences between treated and untreated *Npc1*<sup>-/-</sup> mice, even though CD is thought to be excreted mainly intact in urine [23] (Fig. S2A). H&E staining of kidney also revealed no obvious changes with CD treatment in either *Npc1*<sup>-/-</sup> or WT mice (Fig. S3A). Cholesterol labeling of the liver of untreated *Npc1*<sup>-/-</sup> mice occurred primarily in hepatocytes, with treated mice showing a shift in cholesterol sequestration to apparent Kupffer cells, a change also observed when comparing WT untreated and treated mice (Fig. S2B). Large, lipid-laden hepatocytes were seen in the untreated *Npc1*<sup>-/-</sup> liver tissue but were largely undetectable in CD-treated *Npc1*<sup>-/-</sup> liver as visualized with H&E staining (Fig. S3B). A parallel biochemical study of liver lipids revealed a near normalization of the levels of unesterified cholesterol and sphingomyelin, as well as of bis(monoacylglycerol)phosphate (BMP) and neutral glycolipids (glucosylceramide and lactosylceramide) in chronic CD-treated *Npc1*<sup>-/-</sup> mice (Fig. S4). Filipin analysis of lung revealed cholesterol laden cells in both untreated and CD-treated age-matched *Npc1*<sup>-/-</sup> mice (87 and 90 days, respectively), with a seemingly greater number of cholesterol positive cells present in the CD-treated animal. Lung from a CD-treated *Npc1*<sup>-/-</sup> mouse at end-stage disease (223 days) had a consolidated appearance with more lipid laden cells and cholesterol deposits throughout the tissue than either of the previous *Npc1*<sup>-/-</sup> mice (Fig. S2C). While H&E staining revealed the occasional presence of macrophages in untreated *Npc1*<sup>-/-</sup> lung, CD-treated *Npc1*<sup>-/-</sup> mouse lung showed more macrophages present and also increasing macrophage infiltration the longer CD-treatment was continued (Fig. S3C). Similar lipid-laden macrophages were not evident in either CD-treated or untreated WT lung.

In addition to starting CD treatment at 7 days of age, some *Npc1*<sup>-/-</sup> mice were treated shortly after weaning (P21–P25; post-weaning treated). Even with this two week delay, benefits similar to those seen in *Npc1*<sup>-/-</sup> mice treated beginning at P7 were still evident (Fig. 4C). Onset of clinical signs in *Npc1*<sup>-/-</sup> mice in which





**Figure 5. Chronic CD treatment study in *Npc1*<sup>-/-</sup> mice.** (A) Filipin labeling of unesterified cholesterol in the neocortex of untreated (end-stage, 78 days old) and CD-treated (start at P21; end-stage, 197 days old) *Npc1*<sup>-/-</sup> mice showed reduced cholesterol accumulation in a CD-treated mouse. (B) Higher magnification of neocortex in same CD-treated *Npc1*<sup>-/-</sup> animal as previous panel, showed presence of neurons with cholesterol accumulation while neighboring cells lacked this storage. (C) Confocal microscopy further revealed that gangliosides and cholesterol appeared to always co-sequester within neurons in an untreated *Npc1*<sup>-/-</sup> mouse (end-stage, 78 days old; upper panel). However, some neocortical neurons in a CD-treated *Npc1*<sup>-/-</sup> mouse (start at P7; end-stage, 182 days old; lower panel) had little to no detectable cholesterol accumulation, yet still exhibited ganglioside storage. Cholesterol (red, visualized with BC Theta), GM2 (blue), and GM3 (green); n denotes nucleus of single neuron shown in each image. (D) IHC of untreated and CD-treated *Npc1*<sup>-/-</sup> mice (same mice as A), revealed less GM2 storage (also GM3, not shown) in the neocortex of a CD-treated mouse. (E) Biochemical analysis of ganglioside levels further corroborated the reduction in GM2 and GM3 seen with IHC. Data from WT and untreated mutant mice represent mean  $\pm$  SD. (F) Ultrastructural analysis of neocortical neurons in untreated and CD-treated *Npc1*<sup>-/-</sup> mice (same mice as C) revealed presence of PCBs in both groups, but CD-treated mice appeared to have fewer of these storage bodies. (G) Western blot analysis of LC3-II in 85 day old untreated and CD-treated (start at P21) *Npc1*<sup>-/-</sup> and WT mice revealed a reduction in LC3-II levels in the CD-treated *Npc1*<sup>-/-</sup> mouse. Images taken at 20X (A), 40X (B), 63X (C), and 10X (D); scale bars 20  $\mu$ m (A, B), 2  $\mu$ m (C), 50  $\mu$ m (D), 1  $\mu$ m (F). doi:10.1371/journal.pone.0006951.g005

CD treatment was started post-weaning was delayed and longevity increased, although these mice did show greater variability than mice started at P7. Even though the median lifespan of these *Npc1*<sup>-/-</sup> mice treated post-weaning was less than that of mice started on treatment at P7, there was no significant difference (median age: CD-treated *Npc1*<sup>-/-</sup> mice starting post-weaning: 149 days; CD-treated *Npc1*<sup>-/-</sup> mice starting at P7: 185 days;  $p < 0.1870$ ). Cholesterol and ganglioside analysis of post-weaning treated *Npc1*<sup>-/-</sup> mice revealed reduction in storage compared to untreated *Npc1*<sup>-/-</sup> controls. This finding was supported by biochemical analysis of gangliosides in mice treated from P21 (Fig. 5E). Post-weaning treatment rescued Purkinje cells, although they still showed cholesterol accumulation similar to the *Npc1*<sup>-/-</sup> mice started on treatment at P7. Overall comparison of post-weaning treatment to treatment initiated at P7 suggests that although the latter seems slightly more effective in ameliorating NPC disease, initiating treatment at later dates still provides significant benefit to *Npc1*<sup>-/-</sup> mice.

### Chronic treatment of *Npc2*<sup>-/-</sup> mice with CD ameliorates storage

Positive results from the CD treatment study in NPC1 disease led us to hypothesize that *Npc2*<sup>-/-</sup> mice might also benefit from treatment, as NPC2 disease is similarly characterized by cholesterol and GSL accumulation and the two NPC proteins may function in the same metabolic pathway [9,10]. *Npc2*<sup>-/-</sup> and WT mice were administered CD following the same protocol used for *Npc1*<sup>-/-</sup> mice receiving chronic, every other day treatment, albeit at slightly different start dates. Onset of ataxic gait was delayed from 8 weeks of age in untreated *Npc2*<sup>-/-</sup> mice to approximately 10 weeks of age in treated *Npc2*<sup>-/-</sup> mice. Weight loss in untreated *Npc2*<sup>-/-</sup> mice began at approximately 10 weeks of age, while treated *Npc2*<sup>-/-</sup> mice did not show weight loss until 13 to 14 weeks of age (Fig. 6A). The average lifespan of an untreated *Npc2*<sup>-/-</sup> mouse is approximately 21 weeks of age and treatment with CD increased longevity by 11 weeks, such that on average, end-stage occurred at 32 weeks (Fig. 6B).

Filipin labeling of neocortical neurons in treated *Npc2*<sup>-/-</sup> mice showed reduced accumulation of unesterified cholesterol and IHC analysis of gangliosides revealed less GM2 and GM3 storage (Fig. 6C, D; GM3 not shown). By biochemical quantification (Fig. 6E), when treatment was initiated between P9 and P22, GM2 and GM3 gangliosides levels studied in mice at 137–150 days of age were reduced to about half of those in the untreated mice (212 and 241 nmol/g for GM2, 184 and 216 for GM3 in CD-treated *Npc2*<sup>-/-</sup> mice versus  $413 \pm 42$  and  $401 \pm 34$  nmol/g for GM2 and GM3, respectively in untreated *Npc2*<sup>-/-</sup> mice; mean  $\pm$  SD), and a significant reduction was still sustained in a 272-day old mouse.

Comparison of age-matched CD-treated and untreated *Npc2*<sup>-/-</sup> mice revealed less CD68 labeling in brains of treated animals, suggesting a decrease in neuro-inflammation. Evaluation of end-stage untreated *Npc2*<sup>-/-</sup> mice and age-matched CD-treated *Npc2*<sup>-/-</sup> mice also revealed more surviving Purkinje cells in the cerebellum of treated animals, although all remaining Purkinje cells still exhibited cholesterol accumulation (data not shown). The aforementioned results are analogous to those found in chronically treated *Npc1*<sup>-/-</sup> mice and demonstrate that *Npc2*<sup>-/-</sup> mice also benefit from treatment with CD.

### Effect of CD treatment on free sphingosine levels in NPC mice

In normal tissues free sphingoid bases (essentially sphingosine and sphinganine) are only present in minute amounts, similar to GM2

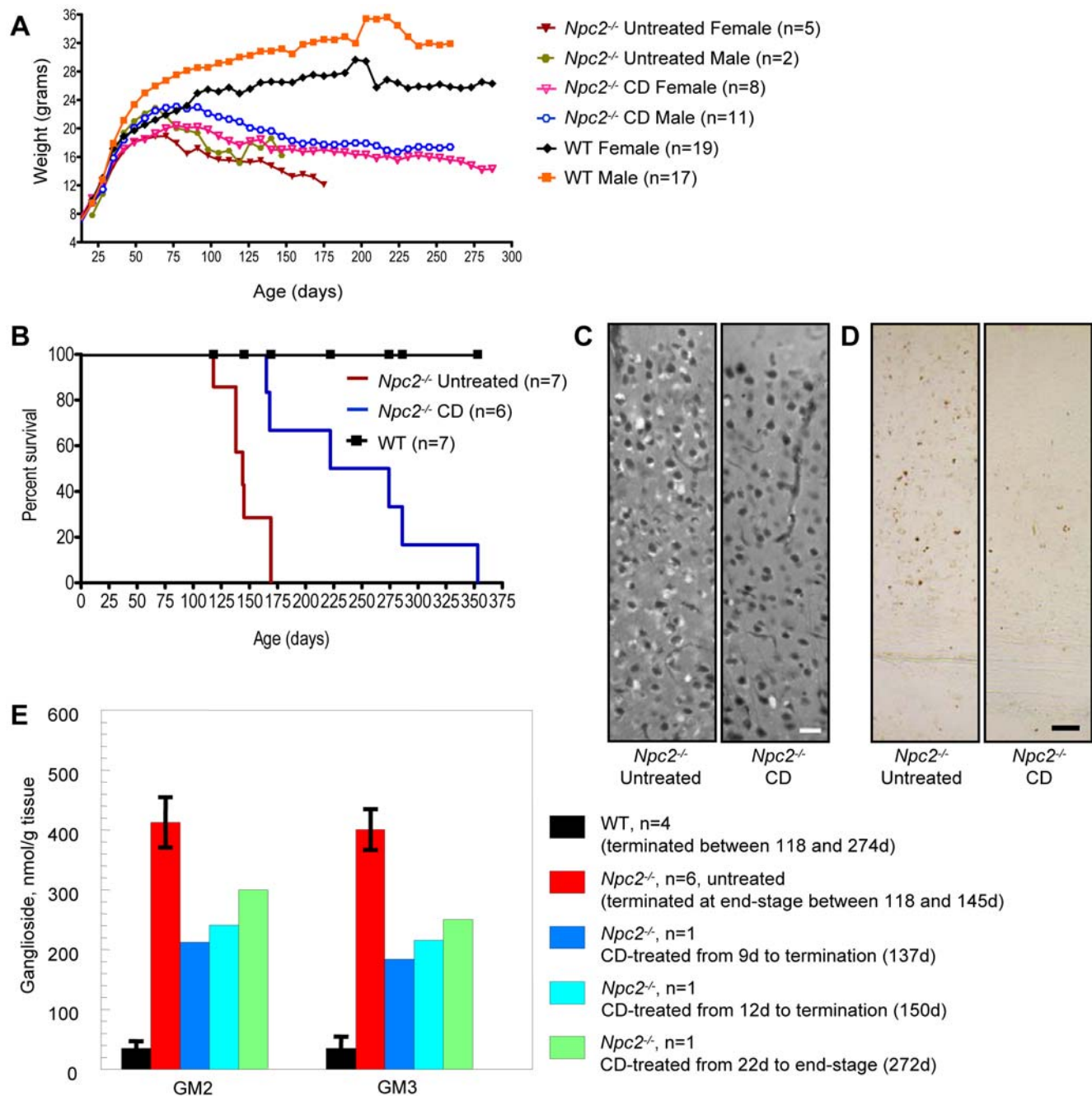
and GM3 gangliosides in the brain. In NPC patients and animal models, sequestration of free sphingosine in lysosomes contributes to the multiple lipid storage pattern, with a many-fold increase in liver, spleen and fibroblasts, but only a modest increase in brain [11,24–25]. Free sphingosine is thus another interesting biomarker of NPC and, due to its free amino group, a putative offending metabolite. Indeed, Platt and colleagues [26] recently postulated that this compound might constitute a major factor of cell dysfunction in NPC. It was therefore also studied in the brains and selected livers of CD-treated and untreated mice. As shown in Fig. 7A, free sphingosine levels, already high in the brain of 22-day old *Npc1*<sup>-/-</sup> mice, increased further to reach a range of 130–180 pmol/mg protein, compared to  $60 \pm 6$  pmol/mg protein in WT mice (mean  $\pm$  SD). Very similar levels were found in brains of *Npc2*<sup>-/-</sup> mice (Fig. 7A). Early chronic CD treatment normalized the free sphingosine level in brain of the 22-day old *Npc1*<sup>-/-</sup> mice, and significantly reduced values were still observed in mice treated from the early post-weaning period. A similar (although less pronounced) trend was found for *Npc2*<sup>-/-</sup> mice. The effect of an early one-week treatment (P7–P14) was, however, not sustained in late stage *Npc1*<sup>-/-</sup> mice, and chronic treatment starting at P81 was inefficient in *Npc2*<sup>-/-</sup> mouse brains. Livers from *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice showed 10–20 fold increased levels of free sphingosine (Fig. 7B), as well as of free sphinganine (data not shown), in good accordance to previously published data [24]. All chronic treatment regimens, even with a late start, appeared efficient in reducing free sphingosine accumulation in liver (Fig. 7B). By contrast, one single week of early treatment from P7 to P14 or P14 to P21 did not result in decreased levels in an end-stage mouse (Fig. 7B), a result consistent with the massive storage of cholesterol and sphingomyelin observed in the livers of those mice (Fig. S4).

### Chronic treatment of MPS IIIA and GM1 gangliosidosis mice with CD does not improve disease state

In addition to NPC disease, several other lysosomal diseases are characterized by accumulation of cholesterol and GSLs [27]. We hypothesized that these diseases might likewise benefit from treatment with CD. Mucopolysaccharidosis type IIIA (MPS IIIA) is caused by a deficiency in sulfamidase, a lysosomal enzyme necessary for the catabolism of the glycosaminoglycan, heparan sulfate [28]. In addition to heparan sulfate storage, neurons are known to accumulate GM2 and GM3 gangliosides and cholesterol [29]. GM1 gangliosidosis is also the result of a lysosomal enzymatic deficiency,  $\beta$ -galactosidase, which catabolizes GM1 to GM2 ganglioside, making GM1 the primary storage material. Cholesterol accumulation also occurs secondarily to that of GM1 in these mice [30] which, however, do not accumulate significant amounts of GM2 and GM3.

Mice with MPS IIIA and GM1 diseases were treated with CD using protocols similar to the NPC studies. Since onset of clinically-evident brain dysfunction is documented to occur later in life for both mouse models (>5 mos) [31–33], analysis was limited to changes in brain storage of cholesterol and gangliosides. Short term studies in the MPS IIIA mice (analogous to the two week study carried out in the NPC1 mice) did not reveal detectable changes in either cholesterol or ganglioside (data not shown). Longer duration, chronic injections of CD also showed no evidence of impact on storage, even when initiated shortly after weaning (P21 or P30) and continued for over 3 months in both models (Fig. 8A–E).

Overall, treatment of both MPS IIIA and GM1 mice with CD showed no detectable benefit in reduction of either cholesterol or GSL storage. These results differed substantially from the ameliorating effects of CD treatment in NPC mice, which included a significant reduction of both cholesterol and GSL accumulation in treated *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice.

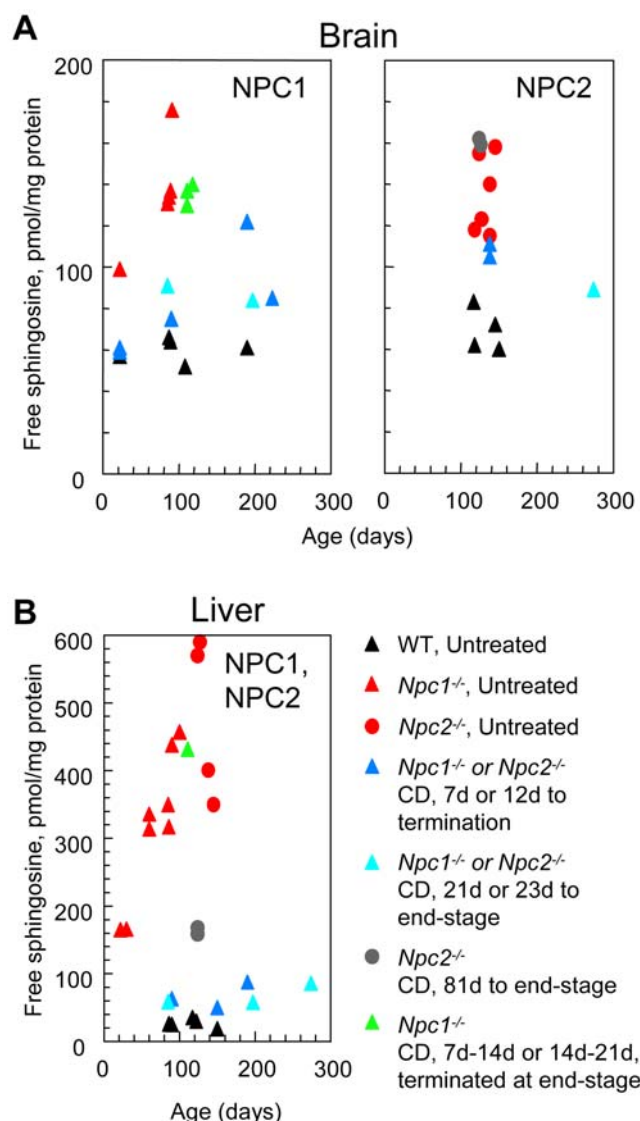


**Figure 6. Chronic CD treatment study in *Npc2*<sup>-/-</sup> mice.** (A) Average weight over time for untreated and CD-treated *Npc2*<sup>-/-</sup> and WT mice. Weights of untreated and CD-treated WT mice were averaged for each gender as there was no significant difference between treatments ( $p < 0.8125$ ). (B) Survival of untreated and CD-treated *Npc2*<sup>-/-</sup> and WT mice. Median survival of *Npc2*<sup>-/-</sup> mice: no treatment, 144 days; CD (every other day), 248 days. CD-treated *Npc2*<sup>-/-</sup> mice lived significantly longer than untreated *Npc2*<sup>-/-</sup> mice ( $p < 0.0108$ ). (C) Filipin labeling of unesterified cholesterol in neocortex of untreated (end-stage, 145 days old) and CD-treated (start at P9; 150 days old) *Npc2*<sup>-/-</sup> mice revealed less cholesterol accumulation in the CD-treated mouse. (D) IHC of the same untreated and CD-treated *Npc2*<sup>-/-</sup> mice showed reduced GM2 labeling in the neocortex of the CD-treated mouse (also for GM3, not shown). (E) Biochemical analysis of ganglioside levels confirmed the reduction in GM2 and GM3 storage seen with IHC. Data from WT and mutant mice represent mean  $\pm$  SD. Images taken at 20X (C) and 10X (D); scale bars 20  $\mu$ m (C) and 50  $\mu$ m (D). doi:10.1371/journal.pone.0006951.g006

## Discussion

The use of combination therapy consisting of MB-DNJ and allopregnanolone in *Npc1*<sup>-/-</sup> mice had a synergistic effect in ameliorating disease progression beyond that seen with either monotherapy. Additionally, we found that treatment with CD, the vehicle for allopregnanolone, also provided benefit. As a result, we

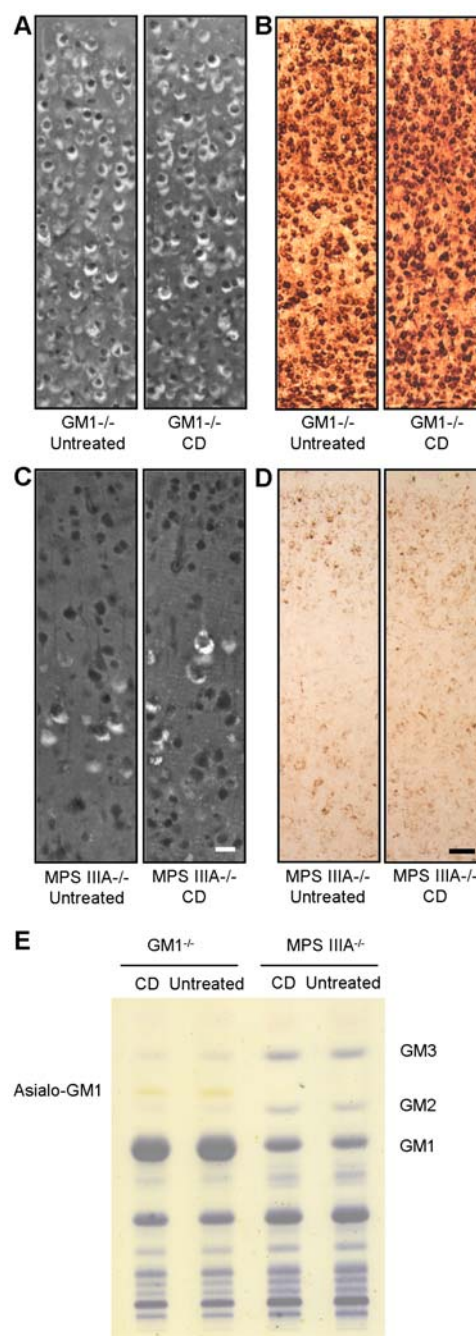
re-examined the proposed beneficial effect of allopregnanolone by utilizing different vehicles or a lower concentration of CD. These studies showed no significant differences in onset of clinical disease, in longevity, or in storage of cholesterol and GSLs between *Npc1*<sup>-/-</sup> mice receiving allopregnanolone in DMSO or corn oil compared to vehicle-only controls. Furthermore, while *Npc1*<sup>-/-</sup> mice treated weekly with allopregnanolone/5% CD or



**Figure 7. Free sphingosine concentrations in NPC disease following CD treatment.** (A) Biochemical analysis of sphingosine in brain of untreated and CD-treated *Npc1*<sup>-/-</sup> and WT mice revealed that chronic CD treatment reduced sphingosine concentrations in affected mice, even when treatment was initiated post-weaning. *Npc2*<sup>-/-</sup> mice exhibited a similar trend, although the effect was less pronounced. (B) Biochemical analysis of liver from untreated and CD-treated *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice showed that with chronic CD treatment, sphingosine accumulation was reduced to levels near those found in WT. doi:10.1371/journal.pone.0006951.g007

5% CD alone did show an increase in lifespan beyond that seen in untreated *Npc1*<sup>-/-</sup> mice, the two groups did not differ significantly when compared with each other. Overall these data indicate that administration of CD (even at low concentrations), has a greater impact on ameliorating disease progression in NPC1 mice than does the administration of allopregnanolone without CD. These findings are further supported by recently published work in which a single injection of allopregnanolone in CD given to *Npc1*<sup>-/-</sup> mice did not increase lifespan beyond those mice receiving only the vehicle, CD [19].

Results from the above studies led us to investigate further the role of CD in ameliorating disease in treated *Npc1*<sup>-/-</sup> mice. Remarkably, *Npc1*<sup>-/-</sup> mice receiving CD at P7 and then every



**Figure 8. Chronic CD treatment study in GM1 and MPS IIIA mice.** (A, B) Filipin labeling of unesterified cholesterol (A) and IHC of GM1 ganglioside (B) in neocortex of untreated (138 days old) and CD-treated (start at P21; 138 days old) mice with GM1 gangliosidosis. Brown punctae indicate GM1 accumulation within cortical neurons. No differences were observed in either filipin or GM1 labeling between CD-treated and untreated GM1 mice. (C, D) Filipin labeling of unesterified cholesterol (C) and IHC of GM2 ganglioside (D) in neocortex of untreated (140 days old) and CD-treated (start at P30; 140 days old) MPS IIIA mice. As with GM1 mice, no differences were observed between CD-treated and untreated MPS IIIA mice. (E) No reductions in GM1, Asialo-GM1, GM2, or GM3 ganglioside levels were seen in cerebral homogenates of CD-treated GM1 and MPS IIIA mice compared to untreated mice as evidenced by the thin-layer chromatography plate. Images taken at 20X (A, C) and 10X (B, D); scale bars 20  $\mu$ m (C, also applicable to A) and 50  $\mu$ m (D, also applicable to B). doi:10.1371/journal.pone.0006951.g008



other day for two weeks revealed little to no intraneuronal accumulation of either cholesterol or gangliosides. Biochemical analysis also showed that these treated mice exhibited ganglioside and sphingosine levels comparable to WT controls. Furthermore, ultrastructural examination of neurons in treated mice revealed few to no PCBs and no other detectable morphological alterations. This apparent normalization of most CNS neurons was also accompanied by expression of the autophagosome marker, LC3-II, at a level resembling WT, while untreated *Npc1*<sup>-/-</sup> animals exhibited a nearly two-fold increase.

Studies examining chronic administration of CD initiated at P7 were found to provide the most significant impact on NPC disease progression. Treated mice showed delayed onset of ataxic gait and tremor, significantly increased lifespan, and greatly reduced accumulation of cholesterol, GSLs, and sphingosine. Treatment with CD also led to the rescue of some cerebellar Purkinje cells outside of lobule X as well as a decrease in a marker of neuroinflammation, CD68, in the brains of treated *Npc1*<sup>-/-</sup> mice. LC3-II levels in the cerebellum of chronically treated *Npc1*<sup>-/-</sup> mice were again decreased when compared to untreated controls and very similar to WT levels, analogous to results of the short term study. Importantly, *Npc1*<sup>-/-</sup> mice administered CD beginning post-weaning (P21–P25) instead of P7 also demonstrated similar beneficial effects. Finally, comparison of CD-treated *Npc1*<sup>-/-</sup> mice using different routes of administration suggested that SC injections were slightly more effective in ameliorating NPC1 disease progression than IP administration. Published research has shown conflicting results in terms of the benefit of CD in amelioration of NPC1 disease [16,19,34]. However, our current results indicate that chronic, every other day treatment with CD, even when initiated post-weaning, proved significantly more beneficial than single [16,19] or weekly injections of CD began at P7 and is the most efficacious of any compound tested to date in delaying NPC disease.

Confocal analysis of cerebral cortex from chronically-treated *Npc1*<sup>-/-</sup> mice revealed that many neurons were essentially devoid of cholesterol storage whereas neighboring cells sometimes exhibited significant accumulation. This finding indicates that CD may impact brain cells differently depending on their type. Interestingly, while some neurons without cholesterol storage also lacked GSL storage, many others displayed numerous GM2 and/or GM3-labeled storage bodies. Cholesterol-sequestering cortical neurons without accompanying ganglioside storage were not observed. What these observations mean in terms of a CD mechanism, or the function of NPC1 and NPC2 proteins, is not presently known. However, the finding is remarkably similar to what was observed in studies in which complex ganglioside synthesis was genetically blocked in NPC1 mice [11,12]. Here, lack of gangliosides other than GM3 and GD3 led to dramatically reduced cholesterol storage in cerebral cortex and other brain areas. Cortical neurons that did persist with cholesterol storage were found to always accumulate GM3 ganglioside, whereas those neurons without GM3 lacked evidence of cholesterol sequestration [12]. While substantial evidence [8,9] supports a direct role for the NPC1 and NPC2 proteins in cholesterol homeostasis, the persistence of ganglioside storage in chronically CD-treated NPC mice, and the apparent dependence of cholesterol sequestration on GM3 in the studies mentioned, continues to raise questions about the overall role of gangliosides in NPC disease in relation to cholesterol sequestration.

Treatment with CD in the NPC1 mouse model has shown significant beneficial effects on CNS neurons, yet the mechanism through which these effects are facilitated is unknown. One important but unresolved issue involves the permeability of the

blood brain barrier (BBB) to CD. CD is a large molecule (molecular weight≈1396 Da) and there are contradictory findings in terms of its penetration of the BBB. *In vivo* studies in which mice were injected intravenously with radiolabeled CD and terminated 1 hour later have suggested that CD does not cross the BBB [34]. Yet a mechanism by which CD could have such striking effects on brain cholesterol and GSL storage in NPC disease without gaining direct access to neurons is difficult to envision. Conceivably, CD may complex with circulating 24S hydroxycholesterol (24S) or other sterols in the bloodstream and in turn create a “sink” which might have the ability to enhance cholesterol egress from brain. Such a mechanism would suggest a feedback loop in which cholesterol homeostasis within the brain could be influenced by levels of 24S or other circulating sterols within the bloodstream, however, no such mechanism is presently known.

Experiments examining cyclodextrins with regard to permeability using an *in vitro* model of the BBB have indicated that a small percentage of CD may be transported across the barrier [35]. Furthermore, there is evidence that use of cyclodextrins as vehicles for pharmacologic agents significantly facilitates their entry into brain [16,36]. If CD does enter the brain, it presumably could exert its impact on cholesterol and GSL accumulation by acting at the plasmalemma of brain cells or after being internalized. One scenario, recently suggested by Dietschy and colleagues, places CD directly in the E/L system of neurons in *Npc1*<sup>-/-</sup> mice where it may act as a substitute for the defective NPC1 protein [19]. This idea implies that after crossing the BBB, HCD is endocytosed by neurons, complexes with cholesterol in LEs/LYs and together with the NPC2 protein facilitates movement of stored cholesterol. However, our studies also indicate that CD treatment is essentially of equal benefit to NPC2-deficient mice. This suggests that CD can replace the NPC1 protein, the NPC2 protein, or an entire cholesterol shuttling mechanism believed controlled conjointly by the two NPC proteins [9]. Perhaps more likely, if CD reaches the E/L system, it can complex with compounds other than cholesterol, for example, BMP or other phospholipids [37] and thereby change E/L membrane dynamics and cholesterol flux. It has been shown, for example, that BMP controls the cholesterol storage capacity of late endosomes [38]. CD may also have the ability to modify the internal environment of the LE/LY compartment in other ways, for example, by altering pH [39], and in some manner facilitate cholesterol flux and/or GSL catabolism. Any of these mechanisms conceivably could lead to normalized trafficking and clearance of accumulated cholesterol and GSLs, but all require CD to enter the neuronal E/L system.

Another scenario to explain the remarkable effect of CD on cholesterol and GSL storage in neurons, but not requiring direct access to the E/L system, would be an interaction with cholesterol at the neuronal plasmalemma. CD is known to have the ability to extract and deplete cholesterol from the cell membranes [23,37], the result of which could be a redistribution of cholesterol from the E/L system to the plasmalemma. This would be consistent with the view that cholesterol accumulation in NPC cells represents a dynamic and mobile storage pool [40], with presumably, redistribution of stored cholesterol allowing secondarily for proper processing of GSLs. Our finding that CD is beneficial in reducing cholesterol and GSLs in neurons lacking either NPC1 or NPC2 proteins, but not in neurons in GM1 gangliosidosis or MPS IIIA, may be revealing of the importance of cholesterol's mobility in the NPC-affected cell compared with other lysosomal diseases.

A third potential mechanism of action for CD might involve a partial or modified BBB penetration. *In vitro* studies examining the effects of cyclodextrins on cholesterol removal from macrophage

foam cells have shown that at low concentrations, CD can act as a shuttle to catalyze the flux of cholesterol between cell membranes and serum lipoproteins and at high concentrations, it can act as a sink for cholesterol [41]. Potentially, CD could simply complex with brain interstitial cholesterol and facilitate its transport into the circulation. CD could also extract cholesterol from the plasma-lemma of endothelial cells lining the BBB and either shuttle cholesterol to acceptor molecules or act as a sink within the bloodstream as discussed earlier. Alternately, a scenario in which CD penetrates the BBB but does not enter the neuron could involve entry into vascular endothelial cells where its effects are exerted from within these frontline BBB cells, or transcytosis across these cells followed by endocytosis into adjacent astrocytes. Astrocytes are known to provide cholesterol to neurons and are poised to exert significant control over cholesterol homeostasis in neuronal cells [42–43]. Conceivably, normalization of cholesterol metabolism within diseased astrocytes could lead to an indirect normalization of cholesterol pools within neurons. Correction of the metabolic defect in astrocytes may then allow neurons to overcome their own metabolic defect, in turn leading to enhanced processing and trafficking of both cholesterol and GSLs. Consistent with this scenario, a recent study in which functional NPC1 protein was expressed in an astrocyte-specific manner in *Npc1*<sup>−/−</sup> mice was reported to lead to increased longevity and decreased neuronal storage of cholesterol [44].

CD is approved for use as a vehicle for drug delivery by several different routes of administration, including parenteral, oral, dermal, and transmucosal [45]. Toxicology studies in animal models, as well as metabolism and pharmacokinetics studies in humans, have shown CD to be well tolerated and any histopathological changes to be reversible [46]. Cyclodextrins are degraded by  $\alpha$ -amylases, but those with substituents on the hydroxyl groups, such as CD, are more resistant to enzymatic degradation [23]. Furthermore, when administered parenterally,  $\beta$ -cyclodextrins are excreted almost completely intact in urine. It is possible that after complexing with cholesterol, CD travels through the bloodstream to the kidneys, where it is excreted in urine still complexed with cholesterol. However there were no noticeable differences in cholesterol accumulation within the kidneys of chronically-treated versus untreated *Npc1*<sup>−/−</sup> mice. Examination of cholesterol storage in livers of treated *Npc1*<sup>−/−</sup> mice revealed a shift from hepatocytes, which exhibit remarkable accumulation in untreated *Npc1*<sup>−/−</sup> mice, to apparent Kupffer cells in the livers of CD-treated mice. The cholesterol accumulation in Kupffer cells of liver from treated *Npc1*<sup>−/−</sup> mice was also noted in liver from treated WT mice. Indeed, the livers of CD treated *Npc1*<sup>−/−</sup> and WT mice looked remarkably similar in terms of cholesterol distribution. Analysis of lungs from untreated and CD-treated *Npc1*<sup>−/−</sup> mice revealed that cellular cholesterol storage was present in both groups. However, lungs from a long-term chronically treated *Npc1*<sup>−/−</sup> mouse at end-stage (approximately 14 weeks older than the *Npc1*<sup>−/−</sup> mice mentioned above) had a consolidated appearance and showed substantial cellular cholesterol accumulation, raising concerns about possible pulmonary complications with long-term CD treatment. These studies, as a whole, suggest that the effects of CD may differ by cell type, for example, when comparing neurons with hepatocytes or macrophage-lineage cells, and that multiple mechanisms of cholesterol mobilization may be involved.

Although the means by which CD exerts its beneficial effects in NPC disease are not understood, the outcome of CD treatment is clearly remarkable. It leads to delay in onset of clinical signs, a significant increase in lifespan, a reduction in cholesterol and ganglioside accumulation in neurons, reduced neurodegeneration,

and normalization of markers for both autophagy and neuro-inflammation. Understanding the mechanism of action for CD will not only provide key insights into the cholesterol and GSL dysregulatory events in NPC disease and related disorders, but may also lead to a better understanding of homeostatic regulation of these molecules within normal neurons. Furthermore, elucidating the role of CD in amelioration of NPC disease will likely assist in development of new therapeutic options for this and other fatal lysosomal disorders.

## Methods

### Animals and drug administration

*Npc1*<sup>−/−</sup> mice, along with WT littermates, were generated by crossing *Npc1*<sup>+/−</sup> males and females in-house. The NPC1 mouse (BALBc/NPC<sup>nh</sup>) was originally obtained from Peter Penchev at the National Institutes of Health (Bethesda, MD). Mouse pups were genotyped according to published protocols [47] and WT and *Npc1*<sup>−/−</sup> mice were enrolled in the combination treatment study. Starting at P7 and weekly thereafter, some mice were injected SC with either 20% CD (control; 4000 mg/kg; H107, Sigma Aldrich, St. Louis, MO) or allopregnanolone (dissolved in 20% CD; 25 mg/kg; P3800-000, Steraloids, Newport, RI). Additionally, some mice were injected IP every day starting at P10 until P23 with either saline (control; 0.9% normal saline; 104 6816, Fisher Scientific, Waltham, MA) or miglustat (dissolved in saline; 300 mg/kg; a gift from Oxford GlycoSciences, Abingdon, UK/Celltech UK, Slough, Berkshire, UK). Following weaning at P23, miglustat and combination treated mice were housed individually in cages and fed powdered chow (Lab Diet 5058, PMI Nutrition International, LLC, Brentwood, MO) to which miglustat was added daily (1200 mg/kg) [14]. *Npc1*<sup>−/−</sup> mice, along with WT controls, were terminated when mice had at least two of the three signs considered to be end-stage disease. Clinical signs of end-stage disease included hunched posture and reluctance to move about the cage, inability to remain upright when moving forward, and weight loss greater than 30% of peak weight (mean end-stage weight = 14.3 g). *Npc1*<sup>−/−</sup> and WT mice placed in the CD treatment studies were administered SC or IP injections of 20% CD (4000 mg/kg) beginning at either P7 or shortly after weaning (P21–P25). Injections were continued every other day until sacrifice. *Npc2*<sup>−/−</sup>, MPS IIIA, and GM1 mice, along with WT littermates, were generated by crossing heterozygotes from in-house colonies of each disease model. Like NPC1 mice, the NPC2 mouse model is also on a uniform background [BALB/c; 10]. The MPSIIIA model is of mixed genetic background, consisting mainly of C57BL/6 with contributions from 129SvJ, CD1, and SJL mouse strains [32]. The GM1 colony also has a mixed genetic background with the major contributing strain being C57BL/6 [31]. These animals were treated with CD using the same protocol as for *Npc1*<sup>−/−</sup> mice, the only variation being the date of first injection, which ranged from P7 to P30.

Treated *Npc1*<sup>−/−</sup> and *Npc2*<sup>−/−</sup> mice, along with WT littermates, were sacrificed at end-stage or with age-matched untreated controls for comparison. MPS IIIA and GM1 mice plus WT littermates were sacrificed after approximately 3 months of treatment with CD, along with untreated controls. Mice were deeply anesthetized with an IP injection of sodium pentobarbital (150 mg/kg) and when insensate, were transcardially perfused with 0.9% saline solution. Following perfusion, a craniotomy was performed and the right cerebrum and right half of cerebellum were removed, along with liver and kidney, which were immediately frozen at −80°C for biochemical analyses. Mice were re-perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB) and additional tissues were



collected (remaining half of brain, liver, kidney, spleen, and lung) and immersion fixed overnight in 4% paraformaldehyde/PB. Tissues were rinsed the following day and stored in PB at 4°C. All animal procedures were carried out according to guidelines approved by the Einstein College of Medicine Institutional Animal Care and Use Committee.

### Statistical analyses

JMP® software (JMP®, Version 7. SAS Institute Inc., Cary, NC) was used to analyze weight data from *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice in the treatment studies. There were no significant differences in weight between untreated and CD-treated WT mice, as Repeated Measures ANOVA revealed no main effect of treatment ( $F_{\text{treatment}} = 0.0027$ ;  $p < 0.8125$ ) and no interactions between treatment and time ( $F_{\text{treatment} \times \text{time}} = 0.4812$ ;  $p < 0.2041$ ). There were also no significant differences between untreated and CD-treated WT weights when individual t-tests were performed at each time point. To determine if statistically significant differences between lifespan of treated and untreated groups occurred, a survival analysis using a Log-rank (Mantel-Cox) Test was performed in GraphPad Prism (GraphPad Software version 5.01, San Diego California USA).

### Antibodies and reagents

The following primary antibodies were purchased for use in immunohistochemistry and/or immunofluorescence: anti-GM3 ganglioside mAb (DH2, mouse IgG3, cell culture supernatant; 10-011; GlycoTech); anti-GM1 ganglioside pAb (IgG, serum; G2006-11; US Biological, Swampscott, MA); anti-CD68 mAb (rat IgG; MCA1957; AbD Serotec, Raleigh, NC); anti-calbindin mAb (mouse IgG, C9848) (Sigma-Aldrich, St. Louis, MO); and anti-LC3 pAb (rabbit IgG, NB100-2220; NOVUS Biologicals, Littleton, CO). Anti-GM2 ganglioside mAb (Mouse IgM, cell culture supernatant) was produced in-house from the 10–11 hybridoma line provided by Progenics Pharmaceuticals, Inc. (Tarrytown, NY). BC-theta was provided by Dr. Y. Ohno-Iwashita (Cellular Signaling Group, Tokyo Metropolitan Institute of Gerontology, Japan) (Iwamoto, 1997).

The following secondary antibodies and reagents were purchased for use in immunoperoxidase staining: biotinylated goat anti-mouse IgM (BA-2020), biotinylated goat anti-mouse IgG (BA-9200), biotinylated goat anti-rabbit IgG (BA-1000), Vectastain ABC kit (PK-4000), and DAB Peroxidase Substrate Kit (SK-4100) from Vector Laboratories (Burlingame, CA). The following secondary and tertiary antibodies were purchased for use in immunofluorescence: FITC-conjugated goat anti-mouse IgG ( $\gamma$  specific-Fc, 55517) from MP Biomedicals (Solon, OH); Alexa Fluor 633-conjugated goat anti-mouse IgM ( $\mu$  chain, A21046), Alexa Fluor 546-conjugated streptavidin (S11225), and Alexa Fluor 488 Signal-Amplification Kit for FITC-conjugated probes (A11053) from Molecular Probes/Invitrogen (Carlsbad, CA). Filipin complex from *Streptomyces filipinensis* (F9765) was purchased from Sigma-Aldrich (St. Louis, MO). Peroxidase-labeled goat anti-rabbit IgG (PI-1000) was purchased from Vector Laboratories (Burlingame, CA) for use in western blotting.

### Immunohistochemical and filipin staining procedures

Immunoperoxidase staining was carried out according to previously published protocols [29]. Primary antibody dilutions were as follows: anti-GM2 (1:5), anti-GM3 (1:50), anti-GM1 (1:5000), anti-calbindin (1:3000), and anti-CD68 (1:200). Secondary antibody dilutions were 1:200 for biotinylated goat anti-mouse IgM, biotinylated goat anti-mouse IgG, and biotinylated goat anti-rabbit IgG.

Immunofluorescence was carried out according to previously published protocols [29]. Primary antibodies were diluted according to: anti-GM2 (1:5), anti-GM3 (1:5), and BC Theta (8  $\mu\text{g/ml}$ ). Secondary antibodies were diluted as follows: FITC-conjugated goat anti-mouse IgG (1:200), Alexa Fluor 633-conjugated goat anti-mouse IgM (1:300), Alexa Fluor 546-conjugated streptavidin (1:750) and Alexa Fluor 488 Signal-Amplification Kit component A (1:80). To visualize unesterified cholesterol, sections were incubated with filipin complex (0.005% dissolved in DMSO and diluted in PBS) or DMSO (control; diluted in PBS) and labeling was carried out according to previously cited protocols.

### Imaging procedures

Brightfield images were obtained using an upright Olympus AX70 microscope and MagnaFire camera. Images of filipin labeling were obtained on the same Olympus microscope but utilizing settings appropriate for acquisition of fluorescent images. Laser scanning confocal fluorescence images were obtained on a Zeiss 510 Duo V2 system using a 63X oil objective (NA = 1.4) and a zoom setting of 3. A multi-track mode, optimized for each fluorophore combination, was employed to help ensure no channel cross-talk. Digital images were further prepared for presentation using Metamorph software (Molecular Devices) and Adobe Photoshop.

### Electron microscopy

According to published protocols [32], fixed tissues were transferred to 0.1M cacodylate buffer, post-fixed in 2% glutaraldehyde, washed and post-fixed in osmium (1% osmium in 0.1% cacodylate buffer), dehydrated and embedded in Epon-aryldite. Ultrathin sections were cut from the plastic embedded blocks, stained with uranyl acetate and lead citrate, and examined with a Philips CM10 electron microscope.

### Biochemical lipid and protein analyses

Lipid analyses were carried out on frozen cerebral hemispheres and liver. Total lipid extracts were obtained as in Fujita et al. [48]. The procedures for ganglioside isolation and subsequent quantitation were as in previous studies [10,49]. Analysis of free sphingoid bases was carried out by high-performance liquid chromatography as described by Rodriguez-Lafrasse et al. [25] using eicosasphinganine as an internal standard. Separation of  $\alpha$ -phthalaldehyde derivatives was achieved on a 200 $\times$ 4.9 mm, 5  $\mu\text{m}$  Spherisorb ODS2 column (Waters) and monitored by fluorometry.

Western blot analysis of LC3 was carried out according to the following method. Frozen brain tissue was homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1% deoxycholic acid, 0.1% SDS supplemented with protease inhibitor cocktail), centrifuged (15000 rpm) for 30 minutes at 4°C and the supernatants (soluble fraction) were collected. Protein concentrations were determined using a BCA protein assay kit. For immunoblotting, samples were analyzed by SDS-PAGE (16% gels) under reducing conditions and transferred to Immuno-blot PVDF membranes. Membranes were blocked in 1x TBS, 0.1% Tween-20, 5% non-fat dry milk, 1% BSA, followed by incubation with an antibody to LC3 (2  $\mu\text{g/ml}$ ) and subsequent incubation with Peroxidase Labeled anti-Rabbit IgG secondary antibody (1:5000). SuperSignal West Pico Chemiluminescent Substrate was used for protein detection on a KODAK 2000R imaging station. Protein quantification for each sample was performed by densitometric analysis using KODAK imaging software and normalized to actin in the same sample. This analysis

was represented as LC3-II/Actin normalized to the wild-type untreated control.

## Supporting Information

**Figure S1** Route of administration of CD in *Npc1*<sup>-/-</sup> mice. (A, B) Filipin labeling of unesterified cholesterol (A) and IHC of GM2 ganglioside (B) in neocortex of 22 day old *Npc1*<sup>-/-</sup> untreated (left panels) and CD-treated mice injected either SC (middle panels) or IP (right panels) starting at P7. Results indicated that while both routes of administration reduced cholesterol accumulation compared to untreated *Npc1*<sup>-/-</sup> mice, SC injections seemed to be more efficacious than IP injections. Images taken at 20X (A) and 10X (B); scale bars 20  $\mu$ m (A) and 50  $\mu$ m (B).

Found at: doi:10.1371/journal.pone.0006951.s001 (4.05 MB TIF)

**Figure S2** Cholesterol accumulation in visceral tissues from chronic CD treatment study in *Npc1*<sup>-/-</sup> mice. (A, B, C) Filipin labeling of unesterified cholesterol in kidney (A), liver (B), and lung (C) from untreated *Npc1*<sup>-/-</sup> (end-stage, 87 days old; first column), CD-treated *Npc1*<sup>-/-</sup> (age-matched, 90 days old; second column), CD-treated *Npc1*<sup>-/-</sup> (end-stage, 223 days old; third column), untreated (87 days old, fourth column) WT, and CD-treated WT (90 days old; fifth column) mice. No obvious differences were seen between cholesterol accumulation in kidneys of untreated versus CD-treated *Npc1*<sup>-/-</sup> mice (A). Filipin labeling indicated a shift of cholesterol storage from hepatocytes in untreated *Npc1*<sup>-/-</sup> mice to presumptive Kupffer cells in CD-treated *Npc1*<sup>-/-</sup> mice, an observation also noted in CD-treated WT mice (B). Overall, filipin labeling of lung from both untreated and CD-treated *Npc1*<sup>-/-</sup> mice suggested the presence of more cholesterol accumulation than in lung from WT mice (C). Images taken at 20X; scale bar 20  $\mu$ m (C, also applicable to A and B).

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**Figure S3** H&E staining of visceral tissues from chronic CD treatment study in *Npc1*<sup>-/-</sup> mice. (A, B, C) H&E staining of kidney (A), liver (B), and lung (C) from untreated and CD-treated *Npc1*<sup>-/-</sup> and WT mice (same mice as in Fig. S2). No obvious differences were noted in kidney between untreated and CD-treated mice (A). Staining of liver indicated the presence of lipid laden hepatocytes within untreated *Npc1*<sup>-/-</sup> tissue but these were not noted in any other mice (B). While macrophages were present

in lung tissue from both untreated and CD-treated *Npc1*<sup>-/-</sup> mice, CD-treated mice showed increasing macrophage infiltration, especially as CD treatment continued to end-stage disease. Lipid-laden macrophages were not observed in either untreated or CD-treated WT tissue (C). Images taken at 20X; scale bar 20  $\mu$ m (C, also applicable to A and B).

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**Figure S4** Effect of chronic CD treatment on lipid storage in liver of *Npc1*<sup>-/-</sup> mice. (A) Thin layer chromatographic profile of total lipids in liver tissue of *Npc1*<sup>-/-</sup> or WT mice, untreated (Unt'd) or CD-treated for the indicated period, visualized with the anisaldehyde reagent. Results indicate that chronic CD treatment, but not 1 week of early CD treatment (far right lane), normalizes lipid storage (Chol, GlcCer, BMP, and Sph) in *Npc1*<sup>-/-</sup> liver to levels near WT. The amount of lipid extract spotted corresponded to 2 mg wet tissue. Chol: unesterified cholesterol; GlcCer: glucosylceramide; BMP: bis(monoacylglycerol)phosphate; Sph: sphingomyelin.

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## Author Contributions

Conceived and designed the experiments: SUW. Performed the experiments: CDD NFA MCM GS SR MTV. Analyzed the data: CDD KD MTV. Contributed reagents/materials/analysis tools: MTV SUW. Wrote the paper: CDD SUW. Contributed to writing of autophagy sections and editing of paper: MCM. Assisted with microscopy, data analysis, and editing of paper: KD. Major collaborator who contributed importantly to discussion section of paper: DSO. Contributed to writing sections dealing with biochemical analysis of gangliosides and to editing the paper: MTV.

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# Cyclodextrin overcomes deficient lysosome-to-endoplasmic reticulum transport of cholesterol in Niemann-Pick type C cells

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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.

cholesterol esterification | NPC1 and NPC2 proteins | sphingomyelinase

Two cholesterol-binding proteins, NPC1 and NPC2, act in concert to export lipoprotein-derived cholesterol from lysosomes (1, 2). Cholesterol enters lysosomes when cholesteryl ester (CE)-rich LDL enters cells by receptor-mediated endocytosis. Within lysosomes, the CEs of LDL are hydrolyzed by acid lipase, and the liberated cholesterol exits the lysosome en route to the endoplasmic reticulum (ER) and the plasma membrane (3). The dual requirement for NPC1 and NPC2 was discovered through studies of cells from patients with Niemann-Pick type C disease (2). These patients harbor two loss-of-function alleles at one or the other of these loci. As a result, cholesterol is trapped in lysosomes, and this excess lysosomal storage causes death through liver, lung, and brain failure (1). In a mouse model of this disease, toxicity was ameliorated and lifespan was extended by treatment with 2-hydroxypropyl- $\beta$ -cyclodextrin (HPCD), which binds to cholesterol and facilitates its exit from lysosomes even in the absence of NPC1 (4). Within 24 h, HPCD treatment led to a decline in free cholesterol and a marked increase in CE in the liver and brain of these mutant mice. The mechanism of this change is unknown.

Recent studies have begun to clarify the separate roles of NPC1 and NPC2 in the lysosomal export process. NPC2, a soluble 132-aa protein, binds to cholesterol by attaching to the ring structure and the hydrophobic iso-octyl side chain (5–7). In contrast, NPC1 is an intrinsic membrane protein containing 1,278 aa and 13 putative membrane-spanning helices. Its N-terminal domain, referred to as NPC1(NTD), projects into the lysosomal lumen (8, 9). Through recombinant DNA technology, NPC1(NTD) can be prepared as a water-soluble fragment of 240 aa in length. Infante, et al. (6) demonstrated that NPC1(NTD) binds to cholesterol in an orientation opposite to that of NPC2 (10). In NPC1, the 3 $\beta$ -hydroxyl group of cholesterol is buried, and the iso-octyl side chain is exposed. Because of this difference in binding orientation, NPC1(NTD), but

not NPC2, can bind to oxysterols such as 25-hydroxycholesterol (25-HC) that have a polar hydroxyl attached to the side chain (6).

The above *in vitro* findings led to the following working model for the exit of LDL-derived cholesterol from the lysosomes (10). After liberation from LDL, cholesterol is bound immediately by NPC2. This binding shields cholesterol from water and prevents its precipitation. NPC2 carries the cholesterol to the lysosomal membrane, where it transfers cholesterol to NPC1(NTD) without the sterol ever passing through the aqueous phase.

The working model predicts that the actions of NPC1 and NPC2 are restricted to late endosomes and lysosomes, the only locations in which both required proteins coexist at high levels. In earlier studies, Lange, et al. (11) suggested that the movement of cholesterol from endosomes and lysosomes to the plasma membrane is not defective in cells lacking functional NPC1. Moreover, Cruz, et al. (12) reported that the initial movement of LDL-derived cholesterol from the lysosomes to the plasma membrane does not require NPC1, but the subsequent internalization of plasma membrane cholesterol and its recycling back to the plasma membrane do. Neither of these conclusions could be reconciled easily with the above working model for NPC2/NPC1 interaction in lysosomes.

To address some of these questions, we here report a series of studies in which we measured the effects of lipoprotein-derived cholesterol and cellular cholesterol on ER regulatory processes in cells with mutations in NPC1 and NPC2. By using acyl-CoA acyltransferase (ACAT) activity as a measure of ER cholesterol delivery, we show that the movement of cholesterol from the lysosomes to the ER is blocked in NPC1- and NPC2-deficient cells and that this block can be alleviated by the treatment of the cells with HPCD.

## Results

After uptake by receptor-mediated endocytosis, LDL-derived cholesterol is liberated in lysosomes, and a portion is transported to the ER, where it binds to Scap and blocks the proteolytic cleavage of sterol regulatory element-binding proteins (SREBPs) (13). Within the ER, some of this cholesterol is esterified to form CE. The esterification reaction can be monitored by incubating cells with [<sup>14</sup>C]oleate and determining the incorporation of radioactivity into CE (14). Fig. 1*A–C* shows an experiment in which we compared esterification in control human fibroblasts and cells from a subject who is a compound heterozygote for mutations at the NPC1 locus (P237S substitution in one allele and I1061T in the other). The cells were

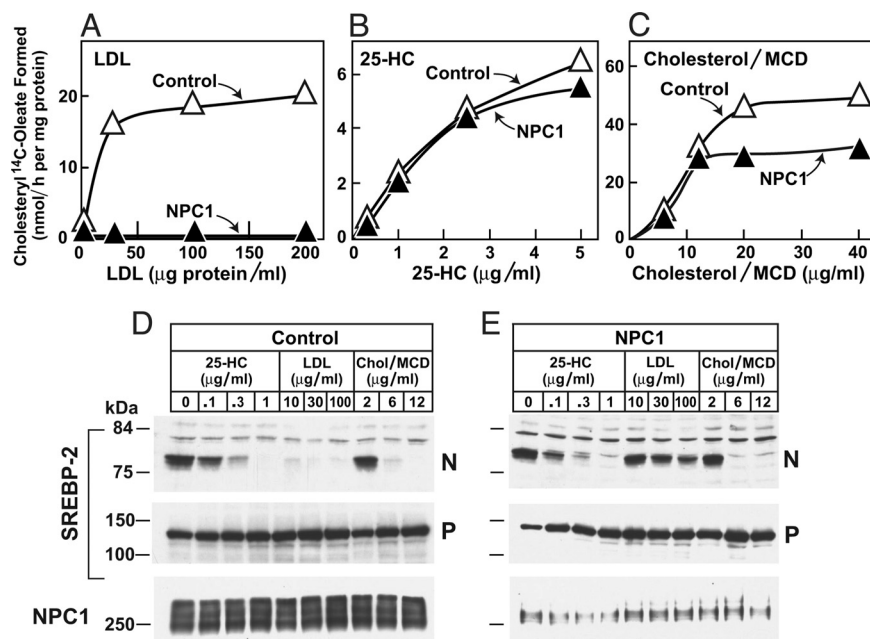
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The authors declare no conflict of interest.

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**Fig. 1.** Role of NPC1 in sterol-mediated cholesteryl ester formation (A–C) and inhibition of SREBP-2 processing (D and E) in control and NPC1 mutant human fibroblasts. On day 0, nontransformed fibroblasts were set up in medium A with 10% FCS at  $2 \times 10^4$  cells per 60-mm dish. On day 3, cells were refed with the same medium, and on day 5, cells were switched to medium A with 10% human lipoprotein-deficient serum (LPDS). (A–C) Cholesterol esterification. On day 7, each dish received medium A containing 10% LPDS, 50  $\mu$ M compactin, 50  $\mu$ M sodium mevalonate, and the indicated concentration of LDL (A), 25-hydroxycholesterol (25-HC) (B), or cholesterol/methyl- $\beta$ -cyclodextrin (MCD) complex (C). After 5 h at 37  $^{\circ}$ C, each monolayer was pulse-labeled for 2 h with 0.2 mM sodium [ $^{14}$ C]oleate-albumin (7,966 dpm/nmol). The cells were harvested for measurement of the content of cholesteryl [ $^{14}$ C]oleate. Each value is the average of duplicate incubations. (D and E) Immunoblot analysis of SREBP-2 processing. On day 7, after incubation for 5 h with the indicated concentration of LDL, 25-HC, or cholesterol/MCD, N-acetyl-leucinal-leucinal-norleucinal was added at a final concentration of 25  $\mu$ g/mL. After 1 h at 37  $^{\circ}$ C, six dishes were harvested and pooled for preparation of nuclear extract and 100,000  $\times$  g membrane fractions, which were analyzed by immunoblotting as described in *SI Materials and Methods*. P, precursor form of SREBP-2; N, cleaved nuclear form of SREBP-2.

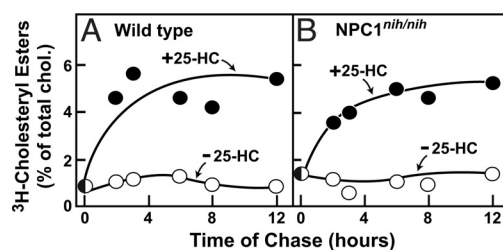
depleted of cholesterol by prior incubation in medium containing lipoprotein-deficient serum. Thereafter, the cells were incubated for 5 h with a source of sterol, after which they were pulse-labeled for 2 h with [ $^{14}$ C]oleate and incorporation into cholesteryl [ $^{14}$ C]oleate was measured. Whereas cholesterol ester synthesis increased markedly in control cells treated with LDL, no such increase was seen in the NPC1 mutant cells (Fig. 1A). This result is similar to that reported by others (15, 16).

In contrast to the difference with LDL, both cell lines responded equally when cholesterol was added in complex with methyl- $\beta$ -cyclodextrin (MCD), which likely delivers cholesterol to cells without passing through lysosomes (Fig. 1C). As shown by Pentchev et al. (15) and Liscum and Faust (16), both cell lines also responded when incubated with 25-HC, which stimulates cholesterol esterification by causing cholesterol to translocate from the plasma membrane to ER (17, 18). A similar set of responses was seen when we measured the inhibition of SREBP-2 cleavage (Fig. 1D and E). In control cells, but not NPC1 mutant cells, LDL blocked SREBP-2 cleavage, resulting in a decline in the nuclear form of SREBP-2. In contrast, cholesterol/MCD and 25-HC blocked SREBP-2 cleavage equally in the two cell lines.

We next tested whether 25-HC can stimulate the esterification of total cell cholesterol in peritoneal macrophages from mice with a homozygous defect in NPC1 (*npc<sup>nihi/nihi</sup>* mice) (Fig. 2A and B). To measure the esterification of total cholesterol, we preincubated wild-type and NPC1 mutant cells with [ $^3$ H]cholesterol for 24 h to radiolabel all cholesterol pools. The [ $^3$ H]cholesterol equilibrates with cellular cholesterol, the vast majority of which is located in the plasma membrane (19, 20). We then washed the cells and incubated them with 25-HC for various times, after which we measured the proportion of cellular [ $^3$ H]cholesterol that had been converted to [ $^3$ H]CE. As shown in Fig. 2A and B, neither cell line esterified its cholesterol in the absence of 25-HC. In the presence of 25-HC,

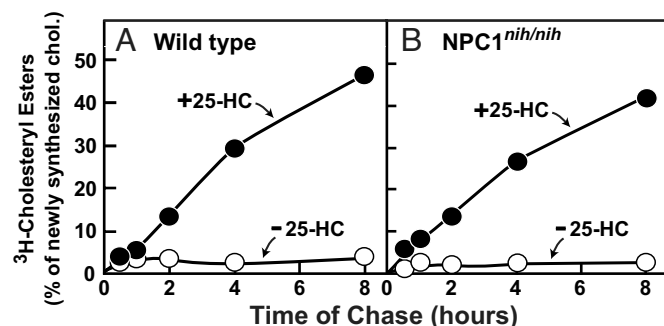
$\approx 5\%$  of total cell cholesterol was esterified within 8 h in both wild-type and NPC1 mutant macrophages. Similar results were obtained in NPC1-deficient hamster cells by Wojtanik and Liscum (21).

To determine whether 25-HC could stimulate the esterification of newly synthesized cholesterol in NPC1 mutant cells, we preincubated cultured fibroblasts from wild-type and *npc<sup>nihi/nihi</sup>* mice with



**Fig. 2.** 25-Hydroxycholesterol (25-HC) stimulated esterification of total cellular cholesterol in macrophages from wild-type and NPC1 mutant mice. On day 0, peritoneal macrophages from wild-type (A) or *npc<sup>nihi/nihi</sup>* mice (B) were set up in medium B with 10% FCS as described in *SI Materials and Methods*. On day 1, the cells were incubated with medium B containing 10% FCS and 8.3 nM [ $^3$ H]cholesterol (132,000 dpm/pmol; added in ethanol at a final concentration of 0.05%). After 24 h at 37  $^{\circ}$ C, each dish was washed with 5 mL of PBS with 0.2% BSA, after which the cells received fresh medium B with 0.2% BSA. After 12 h at 37  $^{\circ}$ C, each monolayer received medium B with 0.2% BSA in the absence or presence of 5  $\mu$ g/mL 25-HC. After incubation for the indicated time, the cells were harvested for measurement of the content of [ $^3$ H]cholesteryl ester (CE) and [ $^3$ H]cholesterol, as described in *SI Materials and Methods*. Each value is the average of duplicate incubations and represents the percentage of [ $^3$ H]cholesterol incorporated into [ $^3$ H]CE relative to [ $^3$ H]cholesterol content at zero time. Zero time values for [ $^3$ H]cholesterol were 10.0 and 14.5 pmol/mg of protein for wild-type and *npc<sup>nihi/nihi</sup>* macrophages, respectively.



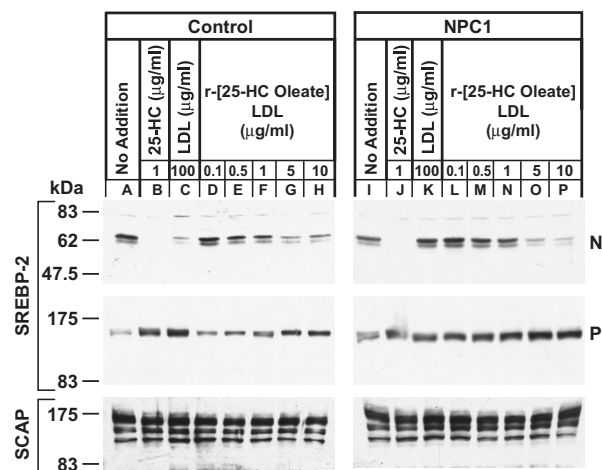


**Fig. 3.** 25-Hydroxycholesterol (25-HC) stimulated esterification of newly synthesized cholesterol in fibroblasts from wild-type and NPC1 mutant mice. On day 0, fibroblasts from wild-type (A) and *npc1<sup>nih/nih</sup>* (B) mice were set up in medium B with 10% FCS and  $4 \times 10^4$  cells per 60-mm dish. On day 3, cells were switched to medium B with 5% newborn calf lipoprotein-deficient serum (LPDS). On day 4, cells were incubated in medium C with 5% LPDS and pulsed-labeled with 167  $\mu$ M [ $^3$ H]acetate (333 dpm/pmol) for 3 h at 14 °C to label newly synthesized cholesterol. After the pulse, each monolayer was washed twice with 3 mL of PBS with 0.2% BSA and then chased at 37 °C with medium B containing 5% LPDS, 50  $\mu$ M compactin (to inhibit cholesterol synthesis), 50  $\mu$ M sodium mevalonate, and 0.1 mM sodium oleate-albumin in the absence or presence of 3  $\mu$ g/mL 25-HC. At the indicated time of chase, the cells were harvested for measurement of [ $^3$ H]cholesteryl ester (CE) and [ $^3$ H]cholesterol. Each value is the average of duplicate incubations and represents the percentage of [ $^3$ H]cholesterol incorporated into [ $^3$ H]CE relative to [ $^3$ H]cholesterol content at zero time. Zero time values for cellular [ $^3$ H]cholesterol were 160 and 192 pmol/mg of protein in wild-type and *npc1<sup>nih/nih</sup>* fibroblasts, respectively.

[ $^3$ H]acetate at 14 °C (Fig. 3 A and B). At this temperature the [ $^3$ H]acetate is incorporated into [ $^3$ H]cholesterol in the ER, but exit from the ER is slowed, leading to a buildup of newly synthesized [ $^3$ H]cholesterol in the ER (22). We then warmed the cells to 37 °C and measured the esterification of this newly synthesized [ $^3$ H]cholesterol. In the absence of 25-HC, none of the [ $^3$ H]cholesterol was esterified in either cell line. In the presence of 25-HC, both cell lines converted >40% of the newly synthesized [ $^3$ H]cholesterol to [ $^3$ H]CE within 8 h (Fig. 3 A and B).

In previous studies, we demonstrated that NPC1(NTD) binds both cholesterol and 25-HC (6). Studies of NPC1 mutants confirmed that cholesterol binding to NPC1(NTD) is necessary for NPC1 to transport cholesterol from the lysosomes to the ER (10). In Fig. 4, we show an experiment designed to determine whether NPC1 is necessary to transport 25-HC from the lysosomes to the ER. We studied fibroblasts from a control subject and a subject with NPC1(P237S/I1061T) that had been immortalized by transfection with a cDNA encoding the catalytic subunit of telomerase, hTERT (23). We extracted all of the cholesterol of LDL and reconstituted the hydrophobic core of the particle with 25-HC oleate. This reconstituted LDL enters cells through the LDL receptor, whereupon the 25-HC oleate is cleaved in lysosomes and the liberated 25-HC suppresses cholesterol synthesis (24). Fig. 4 shows that native LDL failed to block SREBP-2 cleavage in the mutant NPC1 mutant fibroblasts (lane K). In contrast, LDL reconstituted with 25-HC oleate functioned in the mutant cells as well as it did in the control cells (compare lanes D–H and L–P). Thus, 25-HC can exit lysosomes without NPC1. This result is consistent with the findings of Dahl et al. (25), who showed that LDL reconstituted with 25-HC oleate could suppress cholesterol synthesis in NPC1-deficient hamster cells.

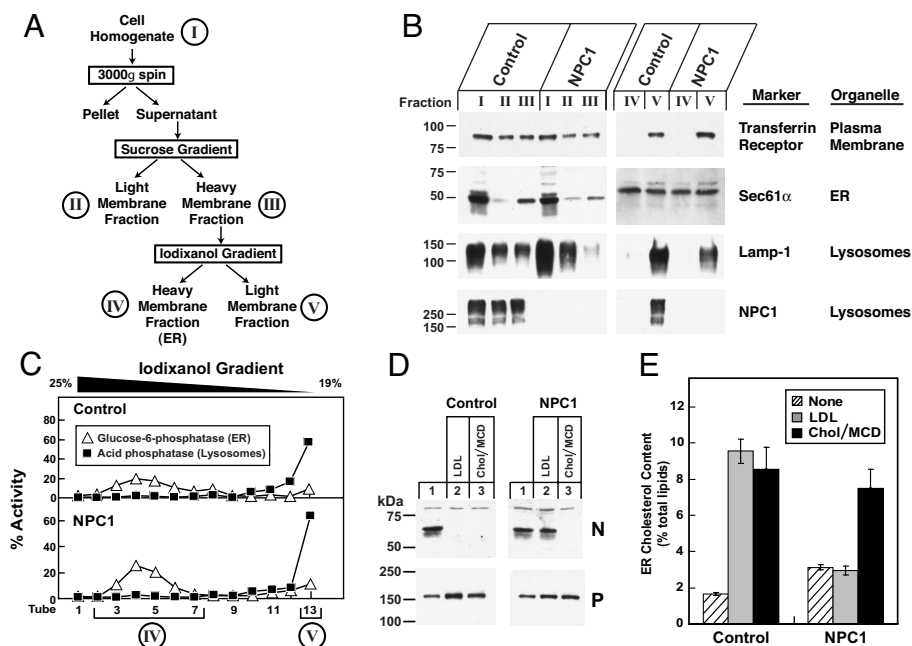
Studies of cholesterol esterification and inhibition of SREBP-2 processing are indirect measures of the concentration of cholesterol in the ER. To measure the concentration of cholesterol in the ER directly, we took advantage of a newly described technique for isolating pure ER membranes from cultured cells (20). In this procedure, cell homogenates are subjected to a two-step gradient ultracentrifugation (Fig. 5A). The heavy membrane fraction from



**Fig. 4.** Inhibition of SREBP-2 processing by LDL-derived 25-hydroxycholesterol (25-HC), but not LDL-derived cholesterol, in NPC1 mutant human fibroblasts. On day 0, hTERT-control and hTERT-NPC1 human fibroblasts were set up in medium A with 10% FCS and  $7 \times 10^4$  cells per 60-mm dish. On day 2, the medium was switched to medium A with 5% human lipoprotein-deficient serum (LPDS). On day 4, the cells were incubated for 5 h in medium A containing 5% LPDS, 50  $\mu$ M compactin, 50  $\mu$ M sodium mevalonate, and one of the following additions: 1  $\mu$ g/mL 25-HC (lanes B, J), 100  $\mu$ g of protein per milliliter of LDL (lanes C, K), or the indicated concentration of LDL reconstituted with 25-HC oleate (r-[25-HC oleate]LDL) (lanes D–H, L–P). Cells then received *N*-acetyl-leucinal-leucinal-norleucinal at a final concentration of 25  $\mu$ g/mL. After a further 1 h at 37 °C, triplicate dishes were harvested and pooled for preparation of nuclear extract and 100,000  $\times$  g membrane fractions, which were analyzed by immunoblotting for SREBP-2 and Scap as described in [SI Materials and Methods](#). P, precursor form of SREBP-2; N, cleaved nuclear form of SREBP-2.

the second gradient (iodixanol fraction IV) contains pure ER membranes as shown by its content of the ER marker Sec61 $\alpha$  and its lack of markers for the plasma membrane (transferrin receptor) and lysosomes (Lamp-1 and NPC1) (Fig. 5B). Fig. 5C shows individual fractions from the iodixanol gradient, illustrating the wide separation between the ER marker glucose-6-phosphatase and the lysosomal marker acid phosphatase. To perform the experiment, hTERT-transformed control human fibroblasts and NPC1 mutant cells were incubated with LDL or with cholesterol complexed to MCD. Fig. 5D shows that both forms of cholesterol blocked the production of nuclear SREBP-2 in control cells, but only the cholesterol/MCD complex had this effect in the NPC1 mutant cells. When control cells were incubated with either LDL or cholesterol/MCD, the cholesterol content of ER membranes rose above the 5% threshold that we showed (20) is necessary to block SREBP-2 processing (Fig. 5E). In the NPC1 mutant cells, LDL failed to increase the ER cholesterol content, whereas cholesterol/MCD caused the normal increase (Fig. 5E).

In earlier studies, we showed that the treatment of cultured cells with sphingomyelinase (SMase) leads to inhibition of SREBP processing and an increase in CE synthesis (26). We postulated that this was caused by the disruption of cholesterol/sphingomyelin complexes in the plasma membrane, with the resultant translocation of the plasma membrane cholesterol to the ER. To measure the concentration of cholesterol in the ER directly and to determine whether NPC1 is necessary for this transport, we performed the experiment shown in Fig. 6. Control hTERT-transformed human fibroblasts or NPC1 mutant cells were incubated at 37 °C for 2 h with SMase. We restricted the incubation to 2 h to guard against any toxicity from the SMase treatment. In the dishes used to measure SREBP-2 cleavage, we included an ACAT inhibitor to block the esterification of cholesterol in the ER. We used  $\beta$ -migrating very low density lipoprotein ( $\beta$ -VLDL) to deliver cholesterol to lyso-



**Fig. 5.** Cholesterol content of purified endoplasmic reticulum (ER) membranes from control and NPC1 mutant human fibroblasts. (A) Endoplasmic reticulum membrane fractionation scheme. (B and C) Immunoblot and enzymatic analysis of membrane fractions. On day 0, 13 dishes of hTERT-control and hTERT-NPC1 fibroblasts were set up in medium A with 10% FCS and  $4 \times 10^5$  cells per 100-mm dish. On day 3, the cells were harvested, and the ER membranes were prepared as described in [SI Materials and Methods](#) and shown in A. Equal volumes of each membrane fraction were subjected to immunoblot analysis for the indicated organelle markers (B) or assayed for the indicated enzyme activity (C). (D and E) Analysis of SREBP-2 cleavage (D) and cholesterol content of purified ER membranes (E). hTERT-control and hTERT-NPC1 fibroblasts (13 dishes for each condition) were set up as described above. On day 2, cells were switched to medium A with 5% human lipoprotein-deficient serum (LPDS). On day 3, cells were switched to medium A with 5% LPDS, 5  $\mu$ M compactin, and 50  $\mu$ M sodium mevalonate. On day 4, the cells received the same medium with 50  $\mu$ M compactin and supplemented with either 100  $\mu$ g of protein per milliliter of LDL or 20  $\mu$ g/mL cholesterol/methyl- $\beta$ -cyclodextrin (MCD) complex as indicated. After incubation for 6 h at 37  $^{\circ}$ C, the cells were harvested. (D) A portion of the harvested cells (5% of total) was fractionated and subjected to immunoblot analysis for SREBP-2. P, precursor form of SREBP-2; N, cleaved nuclear form of SREBP-2. (E) The remaining 95% of the harvested cells were used to purify the ER membranes. Lipids were extracted, and the amount of cholesterol and phospholipids was quantified as described in ref. 20. Cholesterol content in E is expressed as the molar percentage of total lipids (phospholipids + cholesterol). Each bar represents the mean  $\pm$  SEM from five experiments.

somes, because this lipoprotein is richer in cholesterol than LDL. The SMase treatment blocked SREBP-2 cleavage in both the control and the NPC1 mutant cells (Fig. 6A). 25-Hydroxycholesterol also blocked cleavage in both cell lines, but  $\beta$ -VLDL failed to block cleavage in NPC1 mutant cells. As shown in Fig. 6B, SMase treatment increased CE synthesis similarly in control and NPC1 mutant fibroblasts. The SMase elicited the accumulation of similar amounts of cholesterol in the ER in both cell lines (Fig. 6C), a result consistent with previous findings in NPC1-deficient CHO cells (27). These data indicate that NPC1 is not required for the movement of cholesterol from the plasma membrane to the ER in response to SMase treatment.

As mentioned in the Introduction, the treatment of NPC1-deficient mice with cyclodextrin increased CE content in a variety of tissues (4). To determine whether this increase is attributable to ACAT, we studied cells from patients with defects in either NPC1 or NPC2 (Fig. 7). Both of the mutant cell lines failed to show an increase in ACAT-mediated CE synthesis in the presence of LDL (Fig. 7A), but both showed a normal increase in this rate when incubated with 25-HC (Fig. 7B). To measure the effect of HPCD, we preincubated the cells with LDL to load the lysosomes with cholesterol. The cells then were switched to medium with or without 0.1% HPCD. After varying times, the cells were pulse-labeled for 2 h with [ $^{14}$ C]oleate, and incorporation into CE was measured. In control cells, CE synthesis declined after HPCD treatment (open triangles in Fig. 7C and D), likely because HPCD removes cholesterol from cells. In striking contrast, HPCD caused a burst of CE synthesis in both NPC1 and NPC2 mutant cells. The rate of synthesis increased for 10 h and then declined, presumably as the cholesterol from the lysosomes was depleted. Fig. 7D shows

the rate of cholesterol esterification after prior incubation for 6 h with varying concentrations of HPCD. In the NPC1 and NPC2 cells, this rate was maximal at 0.1–0.3% HPCD. Cholesteryl ester synthesis was blocked by the ACAT inhibitor Sandoz 58-035, confirming that it was catalyzed by the ACAT in the ER (Table S1).

## Discussion

The current studies establish several points about the function of NPC1 in human and animal cells: (i) Although NPC1 is essential for egress of lipoprotein-derived cholesterol from lysosomes, it is not required for the egress of lipoprotein-derived 25-HC (Fig. 4); (ii) NPC1 is not required for 25-HC to stimulate the esterification of cellular cholesterol, an event that requires the translocation of cholesterol from the plasma membrane to the ACAT enzyme (Figs. 2 and 3); (iii) NPC1 is not required for the delivery of cholesterol to ER membranes when the cholesterol is added to cells in complex with MCD (Figs. 1 and 5); (iv) NPC1 is not required for the translocation of cholesterol from the plasma membrane to the ER membranes upon treatment with SMase (Fig. 6); and (v) HPCD releases stored cholesterol from the lysosomes of cells with NPC1 or NPC2 mutations, whereupon a portion of the liberated cholesterol is transferred to the ER for esterification (Fig. 7). The latter findings provide a mechanism to explain the finding by Liu et al. (4) that HPCD treatment increased the content of CE in the liver and brain of NPC1 mutant mice.

The ability of LDL-derived 25-HC, but not LDL-derived cholesterol, to exit lysosomes in the absence of NPC1 is likely attributable to the greater water solubility of 25-HC, which allows the 25-HC to diffuse to and through the lysosomal membrane after





As mentioned in the Introduction, several earlier studies suggested that NPC1 is not required for the movement of cholesterol from the lysosomes to the plasma membrane. These studies used cyclodextrins to release cholesterol from the plasma membranes of NPC1-deficient cells. The studies were conducted before the recognition that cyclodextrins release cholesterol from NPC1-deficient lysosomes, and their conclusions thus require reexamination.

Underwood et al. (29) supplied evidence for separate pathways that direct lysosome-derived cholesterol to either the ER or the plasma membrane. The current studies are consistent with this thesis and suggest that HPCD can at least replace NPC1 in directing lysosomal cholesterol to the ER for esterification. Further studies are necessary to delineate the conditions, if any, in which HPCD can direct lysosome-derived cholesterol to the plasma membrane. How HPCD acts at a molecular level remains to be determined.

The ability of HPCD to release cholesterol from NPC1-deficient lysosomes has been exploited already in animal models to show a slowing of organ damage and prolongation of life (4, 30). The data in Fig. 7 suggest that this treatment also will be beneficial in animals that have mutations in NPC2.

## Materials and Methods

**Culture Media.** Medium A is DMEM containing 100 units per milliliter of penicillin and 100  $\mu$ g/mL streptomycin sulfate. Medium B is DMEM (high glucose, 4.5 g/L) containing 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin sulfate. Medium C is medium B without sodium bicarbonate. Where indicated, sterols were added to the medium in an ethanolic solution in which the final ethanol concentration was <0.3%. In Fig. 7, HPCD was dissolved in culture media at the indicated

concentration. The ACAT inhibitor Sandoz 58-035 was dissolved in DMSO and added to the medium at a final concentration of 0.7% (vol/vol).

**Cell Culture.** Nontransformed skin fibroblasts from control subjects, from a patient with NPC1 disease (obtained from American Type Culture Collection, No. GM3123; compound heterozygote for mutations P237S and I1061T), and from a patient with NPC2 disease (obtained from Coriell Cell Repositories, No. GM18455; compound heterozygote for mutations E20X and C47F) were grown in a monolayer at 37 °C in 5% CO<sub>2</sub> and maintained in medium A with 10% (vol/vol) FCS.

Control and NPC1(P237S/I1061T) human fibroblasts were immortalized with the catalytic subunit of human telomerase (hTERT) as described in ref. 23. These cells, designated hTERT-control and hTERT-NPC1, were grown in a monolayer at 37 °C in 5% CO<sub>2</sub> in medium A with 10% (vol/vol) FCS.

Primary skin fibroblast cultures from a wild-type BALB/c mouse and a mutant BALB/c *npc<sup>nlh/nlh</sup>* mouse were established as described in ref. 23. The cells were grown in a monolayer at 37 °C in 5% CO<sub>2</sub> in medium B with 10% (vol/vol) FCS.

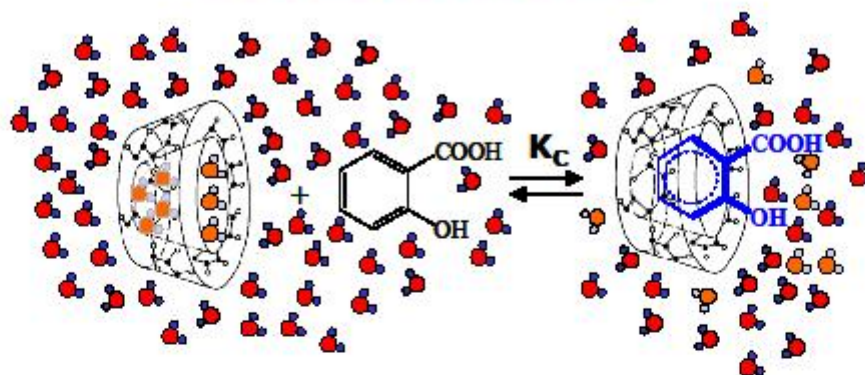
Peritoneal macrophages were prepared from littermate wild-type BALB/c and mutant homozygous BALB/c *npc<sup>nlh/nlh</sup>* mice (31). Heterozygotes for breeding were provided by Stephen Turley and John Dietschy (University of Texas Southwestern Medical Center, Dallas, TX). Macrophages were harvested from the peritoneum of mice 3 days after i.p. injection of 2.5 mL of a 3% (wt/vol) thioglycollate solution and cultured as described in ref. 32. Cells were resuspended in medium B with 10% (vol/vol) FCS at a final density of  $2 \times 10^6$  cells per 35-mm dish and grown in a monolayer at 37 °C in 5% CO<sub>2</sub>.

**Other Methods.** Additional information is described in *SI Materials and Methods*.

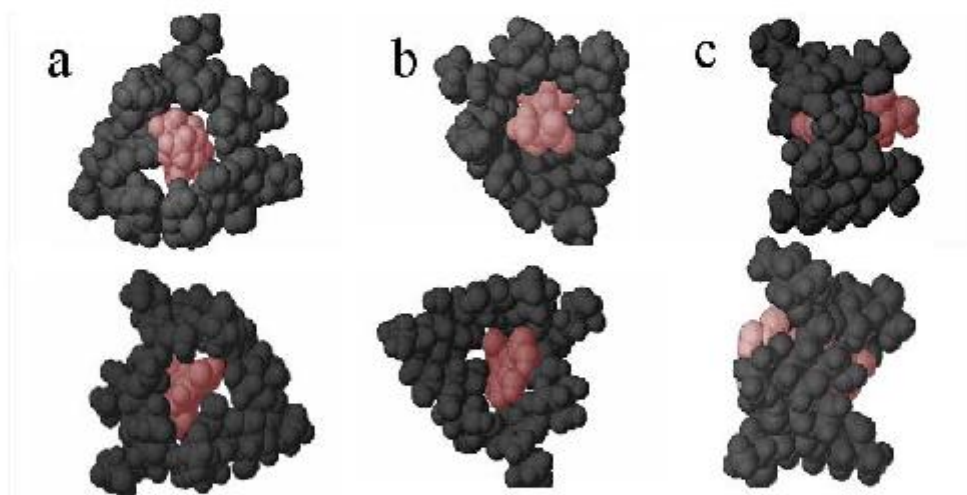
**ACKNOWLEDGMENTS.** We thank Dorothy Goddard and Lisa Beatty for excellent technical assistance, Ijeoma Onwuneme for invaluable help with tissue culture, and Dr. Jeff McDonald for HPLC measurements. This work was supported by grants from the National Institutes of Health (HL20948) and the Perot Family Foundation. L.A.-M. and R.E.I. are supported by the Ara Parsegian Medical Foundation. R.E.I. also is supported by Medical Scientist Training Program Grant 5T32GM08014.

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## CYCLODEXTRINS



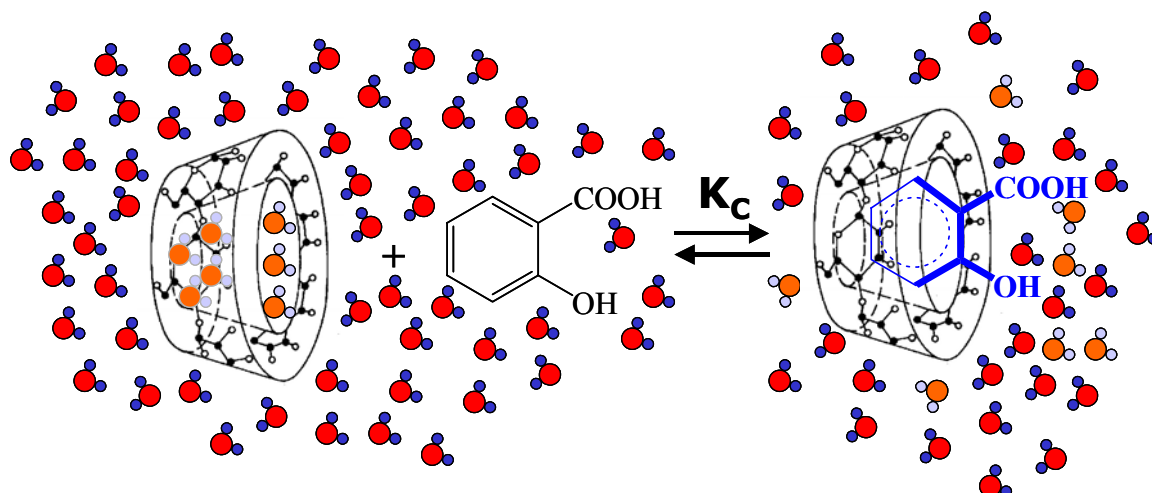
The conventional model of drug/cyclodextrin complex formation (salicylic acid/ $\beta$ -cyclodextrin inclusion complex).



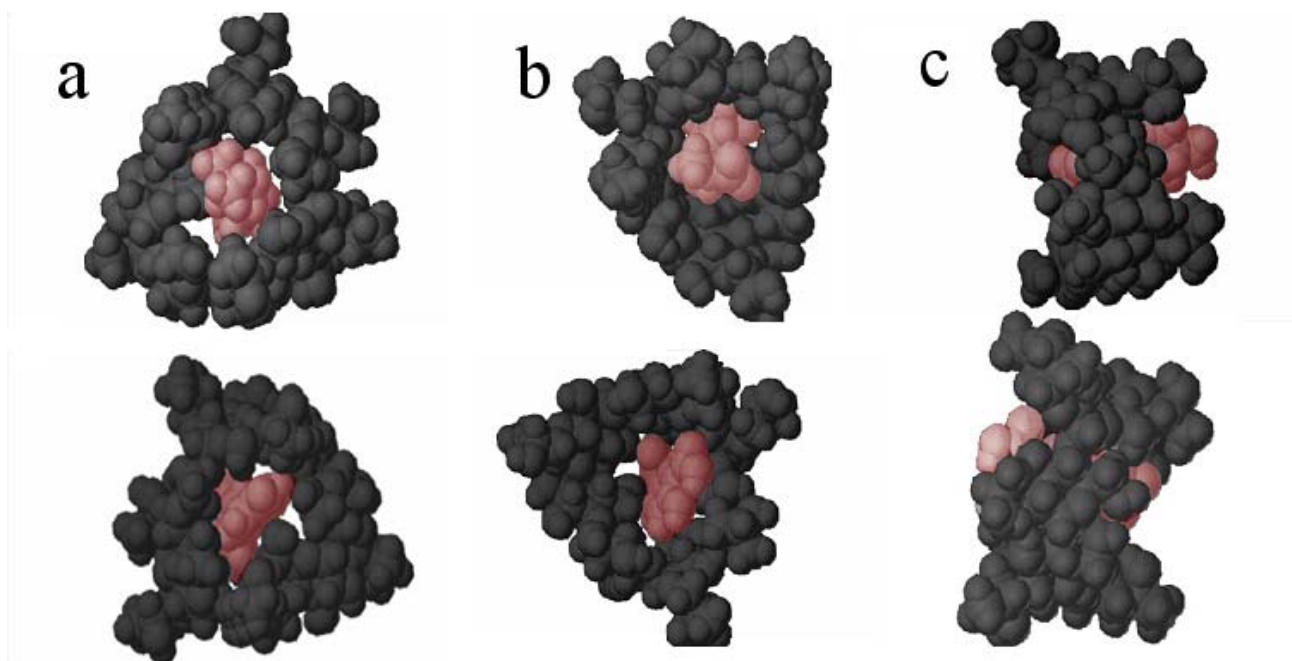
Molecular modeling of diflunisal (below) and ibuprofen (above) in 2-hydroxypropyl- $\beta$ -cyclodextrin cavity in the gas phase seen from the narrow end (a), the wider end (b) and from the side (c) of the cyclodextrin molecule.

A. Magnúsdóttir, M. Másson and T. Loftsson, *J. Incl. Phenom. Macroc. Chem.* **44**, 213-218. 2002.

# CYCLODEXTRINS



The conventional model of drug/cyclodextrin complex formation (salicylic acid/ $\beta$ -cyclodextrin inclusion complex).



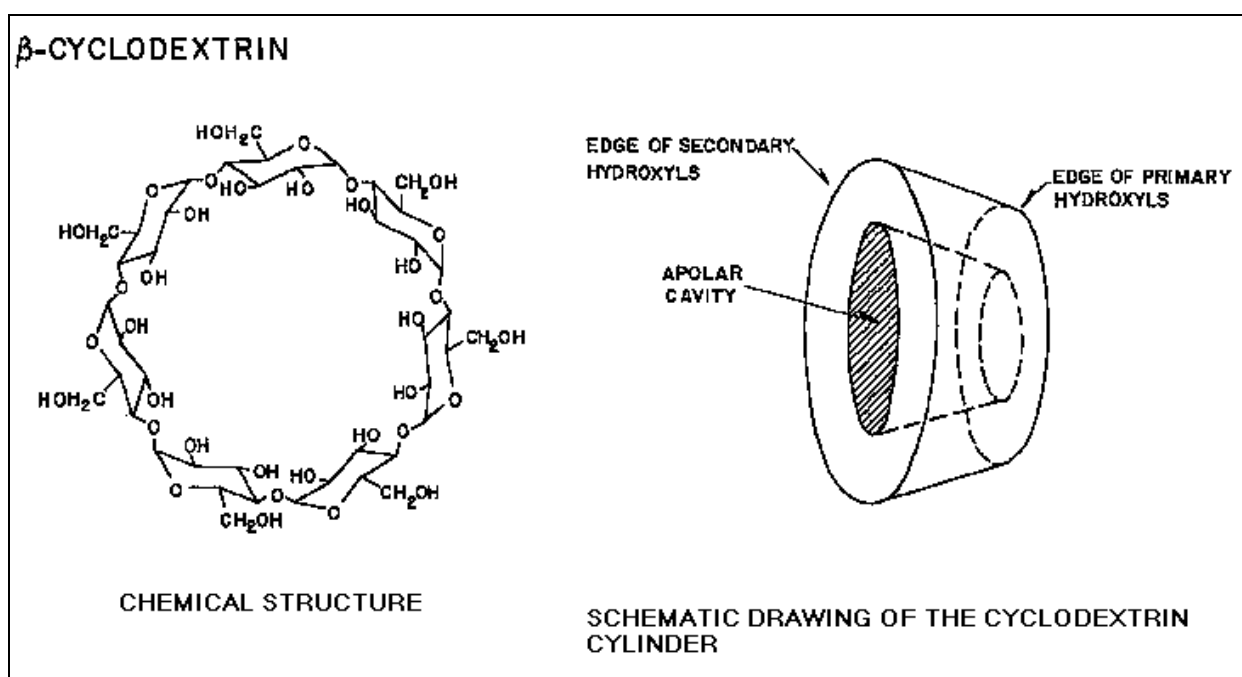
Molecular modeling of diflunisal (below) and ibuprofen (above) in 2-hydroxypropyl- $\beta$ -cyclodextrin cavity in the gas phase seen from the narrow end (a), the wider end (b) and from the side (c) of the cyclodextrin molecule.

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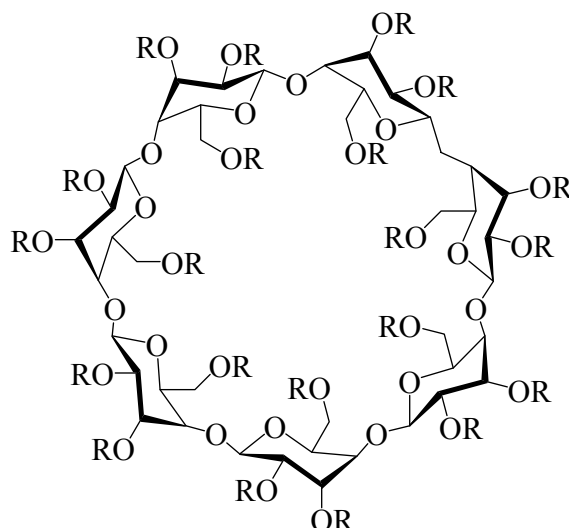


## Structure

Cyclodextrins are a group of structurally related natural products formed during bacterial digestion of cellulose. These cyclic oligosaccharides consist of ( $\alpha$ -1,4)-linked  $\alpha$ -D-glucopyranose units and contain a somewhat lipophilic central cavity and a hydrophilic outer surface. Due to the chair conformation of the glucopyranose units, the cyclodextrins are shaped like a truncated cone rather than perfect cylinders. The hydroxyl functions are orientated to the cone exterior with the primary hydroxyl groups of the sugar residues at the narrow edge of the cone and the secondary hydroxyl groups at the wider edge. The central cavity is lined by the skeletal carbons and ethereal oxygens of the glucose residues, which gives it a lipophilic character. The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution. The natural  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrin consist of six, seven, and eight glucopyranose units, respectively. The natural cyclodextrins, in particular  $\beta$ -cyclodextrin, are of limited aqueous solubility meaning that complexes resulting from interaction of lipophiles with these cyclodextrin can be of limited solubility resulting in precipitation of solid cyclodextrin complexes from water and other aqueous systems. In fact, the aqueous solubility of the natural cyclodextrins is much lower than that of comparable acyclic saccharides. This is thought to be due to relatively strong intermolecular hydrogen bonding in the crystal state. Substitution of any of the hydrogen bond forming hydroxyl groups, even by lipophilic methoxy functions, results in dramatic improvement in their aqueous solubility. Cyclodextrin derivatives of pharmaceutical interest include the hydroxypropyl derivatives of  $\beta$ - and  $\gamma$ -cyclodextrin, the randomly methylated  $\beta$ -cyclodextrin, sulfobutylether  $\beta$ -cyclodextrin, and the so-called branched cyclodextrins such as glucosyl- $\beta$ -cyclodextrin.



**Figure 1.** The chemical structure and the molecular shape of  $\beta$ -cyclodextrin ( $\beta$ CD).



Cyclodextrin	R = H or
$\beta$ -Cyclodextrin	-H
2-Hydroxypropyl- $\beta$ -cyclodextrin	$-\text{CH}_2\text{CHOHCH}_3$
Sulfobutylether $\beta$ -cyclodextrin sodium salt	$-(\text{CH}_2)_4\text{SO}_3^- \text{Na}^+$
Randomly methylated $\beta$ -cyclodextrin	$-\text{CH}_3$
Branched $\beta$ -cyclodextrin	Glucosyl or maltosyl group

**Figure 2.** The structure of  $\beta$ -cyclodextrin and some of its derivatives.

**Table I.** Natural cyclodextrins and some of their derivatives that can be found in marketed pharmaceutical products.

Cyclodextrin	Substitution <sup>a</sup>	MW <sup>b</sup>	Solubility in water (mg/ml) <sup>c</sup>	Indicative bulk price <sup>d</sup> (USD/Kg)
$\alpha$ -Cyclodextrin	-	972	145	45
$\beta$ -Cyclodextrin ( $\beta$ CD)	-	1135	18.5	5
2-Hydroxypropyl- $\beta$ -cyclodextrin	0.65	1400	>600	300
Randomly methylated $\beta$ -cyclodextrin	1.8	1312	>500	350
$\beta$ -CD sulfobutyl ether sodium salt	0.9	2163	>500	-
$\gamma$ -Cyclodextrin	-	1297	232	80
2-Hydroxypropyl- $\gamma$ -cyclodextrin	0.6	1576	>500	400

<sup>a</sup> Average number of substituents per glucopyranose repeat unit.

<sup>b</sup> MW in Daltons.

<sup>c</sup> Solubility in pure water at approx. 25°C.

<sup>d</sup> Approximate bulk price given as the price of one kilogram in US dollars.

The natural  $\alpha$ - and  $\beta$ -cyclodextrin, unlike  $\gamma$ -cyclodextrin, cannot be hydrolyzed by human salivary and pancreatic amylases. However, both  $\alpha$ - and  $\beta$ -cyclodextrin can be fermented by the intestinal microflora. Cyclodextrins are both large (MW ranging from almost 1000 to over 2000 Daltons) and hydrophilic with a significant number of H-donors and acceptors and, thus, are not absorbed from the gastrointestinal tract in their intact form. Hydrophilic cyclodextrins are considered non-toxic at low to moderate oral dosages. Lipophilic cyclodextrin derivatives, such as the methylated cyclodextrins, are to some extent absorbed from the gastrointestinal tract in to the systemic circulation and have been shown to be toxic after parenteral administration. Presently, oral administration of methylated  $\beta$ -cyclodextrin is limited by its potential toxicity. About 30 different pharmaceutical products containing cyclodextrins are now on the market worldwide. Some of these products are listed in Table II. In the pharmaceutical industry, cyclodextrins have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, and to increase their bioavailability and stability. In addition, cyclodextrins can be used to reduce or prevent gastrointestinal and ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug-drug or drug-additive interactions, or to convert oils and liquid drugs into microcrystalline or amorphous powders.

**Table II.** Regulatory status of the natural cyclodextrins (2004).

	Food Approval			Pharmacopoeia Monographs		
	US	Europe	Japan	USP/NF	Ph.Eur.	JP
$\alpha$ CD	In Preparation	Planned	Yes	No	Yes	Yes
$\beta$ CD	GRAS	Food Additive	Yes	Yes	Yes	Yes
$\gamma$ CD	GRAS	Pending	Yes	No	In Progress	Yes

The regulatory status of cyclodextrins is evolving.  $\alpha$ -Cyclodextrin and  $\beta$ -cyclodextrin are listed in a number of pharmacopoeia sources including the US Pharmacopoeia, European Pharmacopoeia and Japanese Pharmacopoeia.  $\gamma$ -Cyclodextrin will soon be included in the US Pharmacopoeia and subsequently in the European Pharmacopoeia as well. A monograph for 2-hydroxypropyl- $\beta$ -cyclodextrin has recently appeared in both the European Pharmacopoeia (4<sup>th</sup> edition (suppl. 4.6) and 5<sup>th</sup> edition) and in the USP28/NF23. Other derivatives are not yet compendial but efforts are underway for their inclusion.  $\beta$ -Cyclodextrin and  $\gamma$ -cyclodextrin are also listed in the generally regarded as safe (GRAS) list of the FDA for use as a food additive. Cyclodextrins are relatively new from a regulatory point of view and as such policies on their use is still not standardized. Consensus seems to be building among regulators that cyclodextrins are excipients and not part

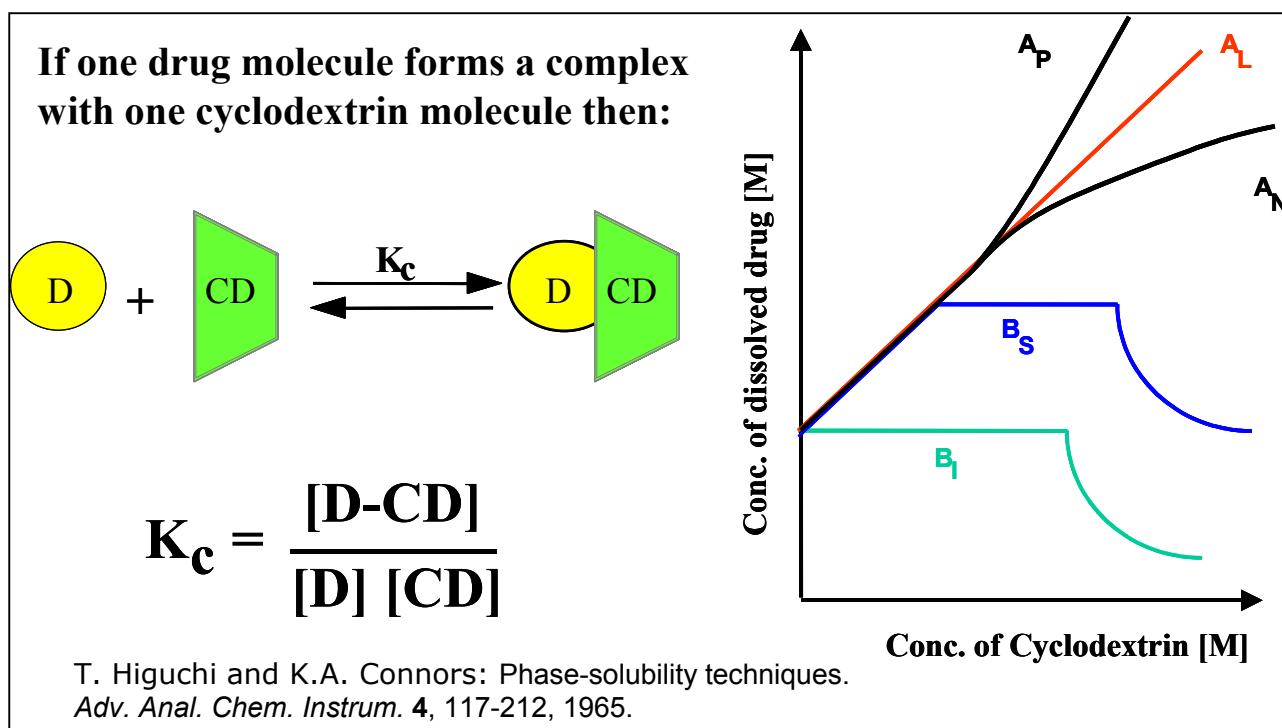
of the drug substance although various opinion have been given and interpretation related to this point can be division and product-specific.

**Table III.** Examples of marketed products containing cyclodextrin.

Drug	Administration route	Trade name	Market
<b><math>\alpha</math>-Cyclodextrin</b>			
Alprostadil (PGE <sub>1</sub> )	IV	Prostavastin	Europe, Japan, USA
Cefotiam hexetil HCl	Oral	Pansporin T	Japan
<b><math>\beta</math>-Cyclodextrin</b>			
Benexate HCl	Oral	Ulgut, Lonmiel	Japan
Dexamethasone	Dermal	Glymesason	Japan
Iodine	Topical	Mena-Gargle	Japan
Nicotine	Sublingual	Nicorette	Europe
Nimesulide	Oral	Nimedex, Mesulid	Europe
Nitroglycerin	Sublingual	Nitropen	Japan
Omeprazol	Oral	Omebeta	Europe
PGE <sub>2</sub>	Sublingual	Prostarmon E	Japan
Piroxicam	Oral	Brexin	Europe
Tiaprofenic acid	Oral	Surgamyl	Europe
<b>2-Hydroxypropyl-<math>\beta</math>-cyclodextrin</b>			
Cisapride	Rectal	Propulsid	Europe
Hydrocortisone	Buccal	Dexocort	Europe
Indomethacin	Eye drops	Indocid	Europe
Itraconazole	Oral, IV	Sporanox	Europe, USA
Mitomycin	IV	Mitozytrex	USA
<b>Randomly methylated <math>\beta</math>-cyclodextrin</b>			
17 $\beta$ -Estradiol	Nasal spray	Aerodiol	Europe
Chloramphenicol	Eye drops	Clorocil	Europe
<b>Sulfobutylether <math>\beta</math>-cyclodextrin</b>			
Voriconazole	IV	Vfend	Europe, USA
Ziprasidone maleate	IM	Geodon, Zeldox	Europe, USA
<b>2-Hydroxypropyl-<math>\gamma</math>-cyclodextrin</b>			
Diclofenac sodium	Eye drops	Voltaren	Europe

### Complex formation and drug solubility

In aqueous solutions cyclodextrins are able to form inclusion complexes with many drugs by taking up a drug molecule, or more frequently some lipophilic moiety of the molecule, into the central cavity. No covalent bonds are formed or broken during the complex formation and drug molecules in the complex are in rapid equilibrium with free molecules in the solution. The driving forces for the complex formation include release of enthalpy-rich water molecules from the cavity, electrostatic interactions, van der Waals interactions, hydrophobic interactions, hydrogen bonding, release of conformational strain and charge-transfer interactions. The physicochemical properties of free drug molecules are different from those bound to the cyclodextrin molecules. Likewise, the physicochemical properties of free cyclodextrin molecules are different from those in the complex. In theory, any methodology that can be used to observe these changes in additive physicochemical properties may be utilized to determine the stoichiometry of the complexes formed and the numerical values of their stability constants. These include changes in solubility, changes in chemical reactivity, changes in UV/VIS absorbance, changes in fluorescence, NMR chemical shifts, changes in drug retention (e.g. in liquid chromatography), changes in pKa values, potentiometric measurements, changes in chemical stability and effects on drug permeability through artificial membranes. Furthermore, since complexation will influence the physicochemical properties of the aqueous complexation media, methods that monitor these media changes can be applied to study the complexation. For example, measurements of conductivity changes, determinations of freezing point depression, viscosity measurements and calorimetric titrations. However, only few of these methods can be applied to obtain structural information on drug/cyclodextrin complexes.



**Figure 3.** Phase-solubility relationships.

Higuchi and Connors have classified complexes based their effect on substrate solubility as indicated by phase-solubility profiles (Figure 3). A-type phase-solubility profiles are obtained when the solubility of the substrate (i.e. drug) increases with increasing ligand (i.e. cyclodextrin) concentration. When the complex is first order with respect to ligand and first or higher order with respect to substrate then A<sub>L</sub>-type phase-solubility profiles is obtained. If the complex is first order with respect to the substrate but second or higher order with respect to the ligand then A<sub>P</sub>-type phase-solubility profiles is obtained. A<sub>N</sub>-type phase-solubility profiles can be difficult to interpret. B-type phase-solubility profiles indicate formation of complexes with limited solubility in the aqueous complexation medium. In general, the water-soluble cyclodextrin derivatives form A-type phase-solubility profiles while the less soluble natural cyclodextrins frequently form B-type profiles. Most drug/cyclodextrin complexes are thought to be inclusion complexes but cyclodextrins are also known to form non-inclusion complexes and complex aggregates capable to dissolve drugs through micelle-like structures. The phase-solubility profiles do not verify formation of inclusion complexes. They only describe how the increasing cyclodextrin concentration influences drug solubility. The most common type of cyclodextrin complexes is the 1:1 drug/cyclodextrin complex (D/CD) where one drug molecule (D) forms a complex with one cyclodextrin molecule (CD):



Under such conditions an A<sub>L</sub>-type phase-solubility diagram, with slope less than unity, would be observed and the stability constant ( $K_{1:1}$ ) of the complex can be calculated from the slope and the intrinsic solubility ( $S_0$ ) of the drug in the aqueous complexation media (i.e. drug solubility when no cyclodextrin is present):

$$K_{1:1} = \frac{Slope}{S_0(1 - Slope)} \quad (2)$$

The value of  $K_{1:1}$  is most often between 50 and 2000 M<sup>-1</sup> with a mean value of 129, 490 and 355 M<sup>-1</sup> for α-, β- and γ-cyclodextrin, respectively (Connors KA. The stability of cyclodextrin complexes in solution. *Chem. Rev.* **97**: 1325-1357 (1997)). For 1:1 drug/cyclodextrin complexes the complexation efficiency (CE) can be calculated from the slope of the phase-solubility diagram:

$$CE = \frac{[D/CD]}{[CD]} = S_0 \cdot K_{1:1} = \frac{slope}{(1 - slope)} \quad (3)$$

When selecting cyclodextrin or complexation conditions during formulation work it can frequently be more convenient to compare the CE than  $K_{1:1}$  values. The most common stoichiometry of higher order drug/cyclodextrin complexes is the 1:2



drug/cyclodextrin complex resulting in A<sub>p</sub>-type phase-solubility diagram. Consecutive complexation is assumed where the 1:2 complex is formed when one additional cyclodextrin molecule forms a complex with an existing 1:1 complex:



The stoichiometry of the system can be probed by curve fitting of the diagram with a quadratic model:

$$S_{\text{tot}} = S_0 + K_{1:1}S_0[\text{CD}] + K_{1:1}K_{1:2}S_0[\text{CD}]^2 \quad (5)$$

Here [CD] represents the concentration of free cyclodextrin but it is customary to plot the total amount of dissolved drug ( $S_{\text{tot}}$ ) against the total amount of cyclodextrin in solution ( $[\text{CD}]_{\text{tot}}$ ) assuming that the extent of complexation is low (i.e.  $[\text{CD}] \sim [\text{CD}]_{\text{tot}}$ ). The value of  $K_{1:2}$  is frequently between 10 and 500 M<sup>-1</sup>, or significantly lower than that of  $K_{1:1}$ .

Various methods can be applied to prepare drug/cyclodextrin complexes, including the solution method, the co-precipitation method, neutralization method, the slurry method, the kneading method, and the grinding method (A. R. Hedges, Industrial applications of cyclodextrins. *Chem. Rev.* **98**: 2035-2044 (1998)). In most cases presence of at least some water is essential for successful complex formation. In solution, cyclodextrin complexes are usually prepared by addition of excess amount of drug to an aqueous cyclodextrin solution. The suspension formed is equilibrated at the desired temperature (which may require periods of up to one week) and then filtered or centrifuged to form clear drug/cyclodextrin complex solution. For preparation of solid complexes, the water is removed from the aqueous drug/cyclodextrin solution by evaporation (e.g. spray-drying) or sublimation (e.g. lyophilization).

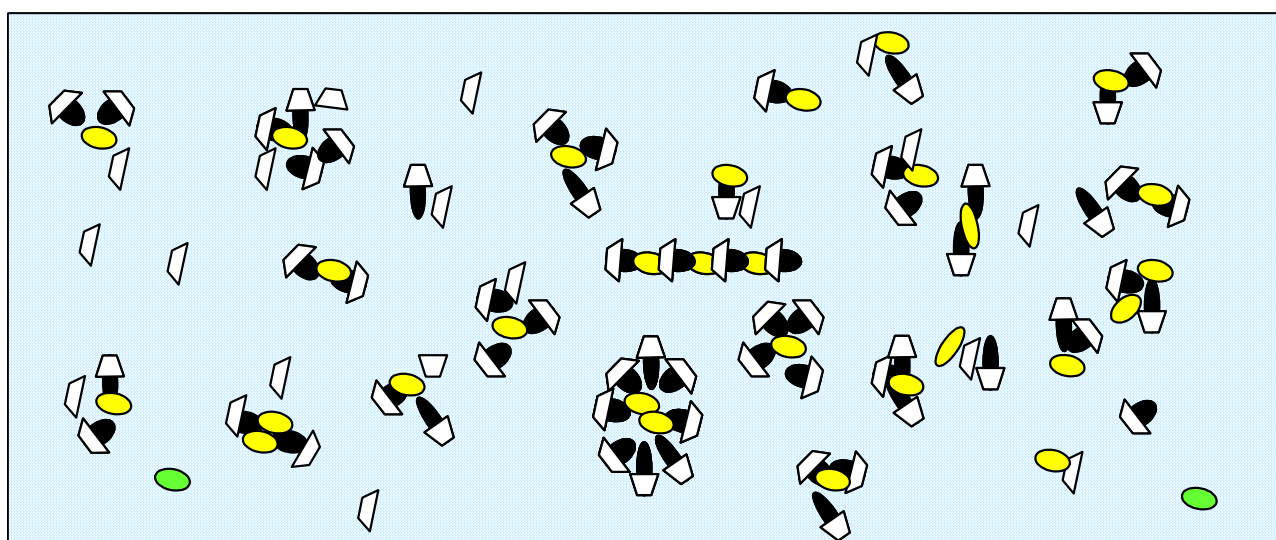
For variety of reasons, such as isotonicity of parenteral formulations and formulation bulk of solid dosage forms, it is important to include as little cyclodextrin as possible in a pharmaceutical formulation. Various methods have been applied to enhance the complexation efficacy (T. Loftsson, M. Másson, J.F. Sigurjónsdóttir, Methods to enhance the complexation efficiency of cyclodextrins. *S.T.P. Pharma Sci.* **9**: 237-242. (1999)). These include addition of polymers to the complexation media, drug ionization and salt formation, addition of hydroxy carboxylic acids to the complexation media, addition of volatile acids or bases to the complexation media, addition of organic salts, and addition of cosolvents (Table IV). However, even under the best conditions, cyclodextrin complexation will result in over four-fold increase in the formulation bulk of solid dosage forms.






**Table IV.** Some methods that can be applied to enhance the complexation efficiency.

Effect	Consequences
Dug ionization	Unionized drugs do usually form more stable complexes than their ionic counterparts. However, ionization of a drug increases its apparent intrinsic solubility resulting in enhanced complexation.
Salt formation	It is sometimes possible to enhance the apparent intrinsic solubility of a drug through salt formation.
Complex-in-complex	It is sometime possible to increase the apparent intrinsic solubility of a drug through formation of metal complexes.
The acid/base ternary complexes	It has been shown that certain organic hydroxy acids (such as citric acid) and certain organic bases are able to enhance the complexation efficiency by formation of ternary drug/cyclodextrin/acid or base complexes.
Polymer complexes	Water-soluble polymers form a ternary complex with drug/cyclodextrin complexes increasing the observed stability constant of the drug/cyclodextrin complex. This observed increase in the value of the constant increases the complexation efficiency.
Solubilization of cyclodextrin aggregates	Organic cations and anions are known to solubilize uncharged drug/cyclodextrin complexes that have limited aqueous solubility. This will enhance the complexation efficiency during preparation of, for example, solid drug/cyclodextrin complex powder.
Combination of two or more methods	Frequently the complexation efficiency can be enhanced even further by combining two or more of the above mentioned methods. For example drug ionization and the polymer method, or solubilization of the cyclodextrin aggregates by adding both polymers and cations or anions to the aqueous complexation medium.

### Non-conventional cyclodextrin complexes

It has generally been assumed that the mechanism whereby cyclodextrin exert their effects, especially their augmentation of solubility, is via the formation of non-covalent, dynamic inclusion complexes. This is a model, which regards drug-cyclodextrin interactions as discrete phenomenon and ignores the possible interaction of these complexes with one another. It is becoming increasingly apparent that such assumptions may not be universally applicable or all encompassing. Specifically, there is a growing body of evidence that supports the important contribution of non-inclusion-based aspects for drug solubilization by cyclodextrins including surfactant-like effects and molecular aggregation. This short review attempts to assess the available literature for areas where such non-inclusion mechanisms are apparent and tries to interpret these in the context of a broader working theory as to how cyclodextrin exert their beneficial effects.



-  **Free drug molecule**
-  **Drug in a non-inclusion complex**
-  **Drug in an inclusion complex**
-  **“Empty” cyclodextrin molecule**
-  **Drug/cyclodextrin inclusion complex**

T. Loftsson, A. Magnúsdóttir, M. Másson and J. F. Sigurjónsdóttir, „Self-association and cyclodextrin solubilization of drugs“, *J. Pharm. Sci.* **91**, 2307-2316 (2002).

T. Loftsson, M. Másson and M. E. Brewster, „Self-Association of cyclodextrins and cyclodextrin complexes“, *J. Pharm. Sci.* **93**, 1091-1099 (2004).

# Expert Opinion

1. Introduction
2. Cyclodextrins
3. Formulation with cyclodextrins
4. Expert opinion and conclusion

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## Cyclodextrins in drug delivery

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Cyclodextrins are a family of cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. Cyclodextrin molecules are relatively large with a number of hydrogen donors and acceptors and, thus, in general they do not permeate lipophilic membranes. In the pharmaceutical industry cyclodextrins have mainly been used as complexing agents to increase aqueous solubility of poorly soluble drugs, and to increase their bioavailability and stability. Studies in both humans and animals have shown that cyclodextrins can be used to improve drug delivery from almost any type of drug formulation. However, addition of cyclodextrins to existing formulations without further optimisation will seldom result in acceptable outcome. Currently there are worldwide about 30 different pharmaceutical products containing drug/cyclodextrin complexes on the market.

**Keywords:** absorption, bioavailability, cyclodextrin, drug delivery, formulation, solubilisation, stabilisation

*Expert Opin. Drug Deliv.* (2005) 2(2):xxx-xxx

### 1. Introduction

All drugs must possess some degree of aqueous solubility to be pharmacologically active, and most drugs need to be lipophilic to be able to permeate biological membranes via passive diffusion. How water-soluble a given drug needs to be is determined by its potency (i.e., the dosage size) and type of formulation. For example, in an aqueous eye drop formulation the dose should be soluble in < 50  $\mu$ l (i.e., one drop) of water, but in a parenteral formulation the dose should preferably be soluble in < 5 ml of water, corresponding to solubility > ~ 15 mg/ml for a medium potent drug (i.e., dose of about 1 mg/kg) [1]. Oral absorption of drugs with solubilities < 0.1 mg/ml is likely to be dissolution limited [2]. On the other hand, if a drug is too water soluble (and/or too hydrophilic) the dissolved drug molecule will have little tendency to partition from the aqueous exterior into a lipophilic biomembrane (e.g., the eye cornea or gastrointestinal mucosa) and then to permeate the membrane. High-throughput screening approaches to drug development have led to an increasing number of lipophilic water-insoluble drug candidates [3] or drugs whose clinical usefulness is hampered by their insolubility in water. These drugs are classified as Class II (i.e., poorly soluble/highly permeable) or Class IV (i.e., poorly soluble/poorly permeable) drugs according to the Biopharmaceutics Classification System [4]. In general, formulation techniques that increase the apparent aqueous solubility of Class II and Class IV drugs without decreasing their lipophilicity will enhance their absorption through biological membranes. These techniques include particle size reduction, salt formation, solid dispersion, melt extrusion, spray drying, and complexation, as well as drug solutions in microemulsions, liposomes, and non-aqueous solvents. The following is a review of cyclodextrins and their place in drug delivery.

### 2. Cyclodextrins

Cyclodextrins are natural cyclic oligosaccharides that were discovered > 100 years ago [5], but only recently did highly purified cyclodextrins become available as pharmaceutical excipients. Worldwide about 30 different pharmaceutical products

**Table 1. Some examples of marketed products containing cyclodextrin.**

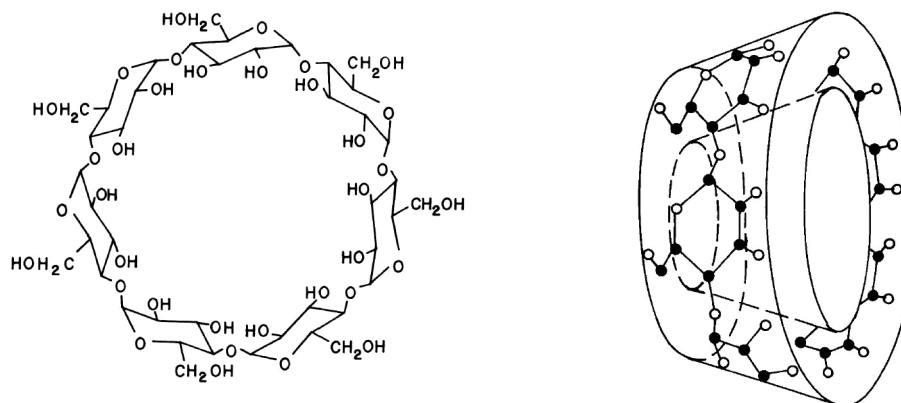
Drug	Formulation	Trade name	Company
<b><i>α-Cyclodextrin</i></b>			
Alprostadil (PGE <sub>1</sub> )	IV solution	Prostavasin	Ono (Japan)
Cefotiam hexetil HCl	Oral tablet	Pansporin T	Takeda (Japan)
<b><i>β-Cyclodextrin</i></b>			
Benexate HCl	Oral capsule	Ulgut	Teikoku Kagaku Sangyou (Japan)
Dexamethasone	Dermal ointment	Glymesason	Fujinaga (Japan)
Nicotine	Sublingual tablet	Nicorette	Pharmacia (Sweden)
Nitroglycerin	Sublingual tablet	Nitrophen	Nihon Kayaku (Japan)
Piroxicam	Oral tablet	Brexin	Chiesi (Italy)
Tiaprofenic acid	Oral tablet	Surgamyl	Roussel-Maestrelli (Italy)
<b><i>2-Hydroxypropyl-β-cyclodextrin</i></b>			
Cisapride	Suppository	Propulsid	Janssen (Belgium)
Indomethacin	Eye drop solution	Indocid	Chauvin (France)
Itraconazole	Oral and IV solutions	Sporanox	Janssen (Belgium)
Mitomycin	IV solution	Mitozytrex MitoExtra	SuperGen (USA) Novartis (Switzerland)
<b><i>Randomly methylated β-cyclodextrin</i></b>			
17β-Oestradiol	Nasal spray	Aerodiol	Servier (France)
Chloramphenicol	Eye drop solution	Clorocil	Oftalder (Portugal)
<b><i>Sulfobutylether β-cyclodextrin</i></b>			
Voriconazole	IV solution	Vfend	Pfizer (USA)
Ziprasidone maleate	IM solution	Geodon, Zeldox	Pfizer (USA)
<b><i>2-Hydroxypropyl-γ-cyclodextrin</i></b>			
Diclofenac sodium	Eye drop solution	Voltaren ophtha	Novartis (Switzerland)

containing cyclodextrins are on the market (Table 1). In the pharmaceutical industry cyclodextrins have mainly been used as complexing agents to increase aqueous solubility of poorly soluble drugs, and to increase their bioavailability and stability. In addition, cyclodextrins can, for example, be used to reduce gastrointestinal drug irritation, convert liquid drugs into microcrystalline or amorphous powder, and prevent drug–drug and drug–excipient interactions. A number of books and review articles have been published on the pharmaceutical applications of cyclodextrins [6–18].

## 2.1 Structure and properties

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. They consist of (α-1,4-)-linked α-D-glucopyranose units with a lipophilic

central cavity (Figure 1). Due to the chair formation of the glucopyranose units, cyclodextrin molecules are shaped like cones with secondary hydroxy groups extending from the wider edge and the primary groups from the narrow edge. This gives cyclodextrin molecules a hydrophilic outer surface, whereas the lipophilicity of their central cavity is comparable to an aqueous ethanolic solution [8]. The most common natural cyclodextrins consist of six (α-cyclodextrin), seven (β-cyclodextrin) and eight (γ-cyclodextrin) glucopyranose units. Although the natural cyclodextrins and their complexes are hydrophilic, their aqueous solubility is rather limited, especially that of β-cyclodextrin. This is thought to be due to relatively strong binding of the cyclodextrin molecules in the crystal state (i.e., relatively high crystal lattice energy) [9]. Random substitution of the hydroxy groups, even by hydrophobic



**Figure 1. The chemical structure and the conical shape of the  $\beta$ -cyclodextrin molecule.** Used with permission from Journal of Pharmaceutical Sciences © (1996) Wiley-Liss, Inc., A Wiley Company [9].

**Table 2. Cyclodextrins that can be found in marketed pharmaceutical products.**

Cyclodextrin	Substitution*	MW (Da)	Solubility in water (mg/ml) <sup>‡</sup>	Applications
$\alpha$ -Cyclodextrin	-	972	145	Oral, parenteral, topical
$\beta$ -Cyclodextrin	-	1135	18.5	Oral, topical
2-Hydroxypropyl- $\beta$ -cyclodextrin	0.65	1400	> 600	Oral, parenteral, topical
Randomly methylated $\beta$ -cyclodextrin	1.8	1312	> 500	Oral <sup>§</sup> , topical
$\beta$ -Cyclodextrin sulfobutyl ether sodium salt	0.9	2163	> 500	Oral, parenteral, topical
$\gamma$ -Cyclodextrin	-	1297	232	Oral, parenteral <sup>§</sup> , topical
2-Hydroxypropyl- $\gamma$ -cyclodextrin	0.6	1576	> 500	Oral, parenteral, topical

\*Average number of substituents per glucopyranose repeat unit. <sup>‡</sup>Solubility in pure water at ~ 25°C. <sup>§</sup>In very limited amounts. MW: Molecular weight.

moieties such as methoxy functions, will result in dramatic improvements in their solubility (Table 2). The main reason for the solubility enhancement is that the random substitution transforms the crystalline cyclodextrins into amorphous mixtures of isomeric derivatives. Cyclodextrin derivatives of pharmaceutical interest include the hydroxypropyl derivatives of  $\beta$ - and  $\gamma$ -cyclodextrin, the randomly methylated  $\beta$ -cyclodextrin, sulfobutylether  $\beta$ -cyclodextrin, and the so-called branched cyclodextrins such as glucosyl- $\beta$ -cyclodextrin.

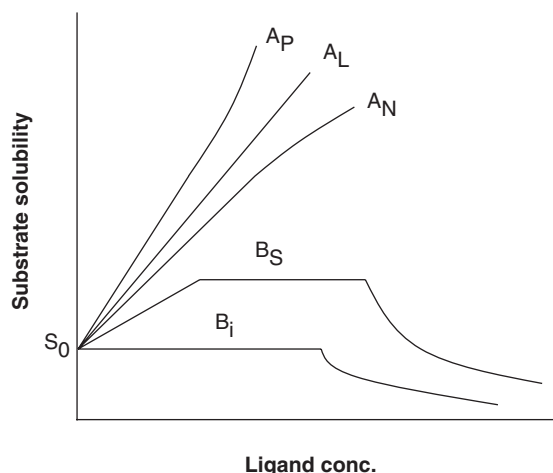
Cyclodextrin molecules are relatively large (molecular weight ranging from almost 1000 to > 2000 Da) with a large number of hydrogen donors and acceptors, and are consequently poorly absorbed through biological membranes. The natural  $\alpha$ - and  $\beta$ -cyclodextrin, unlike  $\gamma$ -cyclodextrin, cannot be hydrolysed by human salivary and pancreatic amylases [19,20], but all three are subjected to fermentation by the intestinal microflora. Hydrophilic cyclodextrins are non-toxic at low to moderate oral dosages [11,15]. The natural cyclodextrins and their derivatives are used in topical and oral formulations, but only  $\alpha$ -cyclodextrin and the hydrophilic derivatives of  $\beta$ - and  $\gamma$ -cyclodextrin can be used in parenteral formulations.  $\gamma$ -Cyclodextrin forms visible aggregates in aqueous solutions and, thus, is not well suited for parenteral formulations [21]. Due to its

nephrotoxicity,  $\beta$ -cyclodextrin cannot be used in parenteral formulations. Lipophilic cyclodextrin derivatives, such as the methylated cyclodextrins, are to some extent absorbed from the gastrointestinal tract into the systemic circulation and have been shown to be toxic after parenteral administration [11]. Presently, oral administration of methylated  $\beta$ -cyclodextrin is limited by its potential toxicity. Cyclodextrin monographs can be found in several Pharmacopoeias. For example,  $\alpha$ -cyclodextrin and  $\beta$ -cyclodextrin are listed in the US Pharmacopeia, European Pharmacopeia and the Japanese Pharmacopeia.  $\gamma$ -Cyclodextrin will soon be included in the US Pharmacopeia and subsequently in the European Pharmacopeia as well. A monograph for 2-hydroxypropyl- $\beta$ -cyclodextrin has recently appeared in the European Pharmacopeia.  $\beta$ -Cyclodextrin and  $\gamma$ -cyclodextrin are also listed in the 'generally regarded as safe' list of the FDA for use as food additives.

## 2.2 Complex formation and drug solubility

In aqueous solutions cyclodextrins are able to form inclusion complexes with many drugs by taking up a drug molecule, or more frequently some lipophilic moiety of the molecule, into the central cavity. No covalent bonds are formed or broken during the complex formation, and drug molecules in the



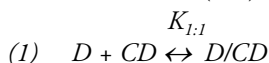


**Figure 2. Phase-solubility profiles and classification of complexes according to Higuchi and Connors [26].**  $S_0$  is the intrinsic solubility of the substrate (the drug) in the aqueous complexation medium when no ligand (cyclodextrin) is present.

complex are in rapid equilibrium with free molecules in the solution. The driving forces for the complex formation include release of enthalpy-rich water molecules from the cavity (i.e., water molecules that cannot have a full complement of hydrogen bonds), electrostatic interactions, van der Waals' interactions, hydrophobic interactions, hydrogen bonding, release of conformational strain and charge-transfer interactions [9,22]. The physicochemical properties of free drug molecules are different from those bound to the cyclodextrin molecules. Likewise, the physicochemical properties of free cyclodextrin molecules are different from those in the complex. In theory, any methodology that can be used to observe these changes in additive physicochemical properties may be utilised to determine the stoichiometry of the complexes formed and the numerical values of their stability constants [23-25]. These include changes in solubility, chemical reactivity, UV/VIS absorbance, fluorescence, drug retention (e.g., in liquid chromatography), pKa values, potentiometric measurements and chemical stability, nuclear magnetic resonance (NMR) chemical shifts and effects on drug permeability through artificial membranes. Furthermore, because complexation will influence the physicochemical properties of the aqueous complexation media, methods that monitor these media changes can be applied to study the complexation; for example, measurements of conductivity changes, determinations of freezing point depression, viscosity measurements and calorimetric titrations. However, only few of these methods can be applied to obtain structural information on drug/cyclodextrin complexes.

Higuchi and Connors [26] have classified complexes based their effect on substrate solubility as indicated by phase-solubility profiles (Figure 2). A-type phase-solubility profiles are obtained when the solubility of the substrate (i.e., drug) increases with increasing ligand (i.e., cyclodextrin) concentration. When the

complex is first order with respect to ligand and first or higher order with respect to substrate then  $A_L$ -type phase-solubility profiles are obtained. If the complex is first order with respect to the substrate, but second or higher order with respect to the ligand then  $A_P$ -type phase-solubility profiles are obtained.  $A_N$ -type phase-solubility profiles can be difficult to interpret. The negative deviation from linearity may be associated with cyclodextrin-induced changes in the dielectric constant of the aqueous complexation media, changes in complex solubility or self-association of cyclodextrin molecules [24]. B-type phase-solubility profiles indicate formation of complexes with limited solubility in the aqueous complexation medium. In general, the water-soluble cyclodextrin derivatives form A-type phase-solubility profiles, whereas the less soluble natural cyclodextrins frequently form B-type profiles. Most drug/cyclodextrin complexes are thought to be inclusion complexes, but cyclodextrins are also known to form non-inclusion complexes and complex aggregates capable of dissolving drugs through micelle-like structures [27,28]. The phase-solubility profiles do not verify formation of inclusion complexes. They only describe how the increasing cyclodextrin concentration influences drug solubility. To distinguish between inclusion and non-inclusion complexes, experimental results from phase-solubility studies have to be compared with other experimental results from, for example, UV/VIS, fluorescence and NMR studies [27,28]. The most common type of cyclodextrin complex is the 1:1 drug/cyclodextrin complex (D/CD) in which one drug molecule (D) forms a complex with one cyclodextrin molecule (CD):



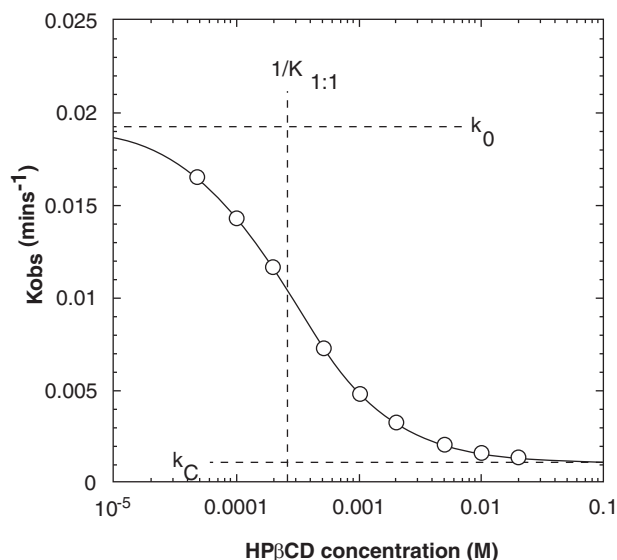
Under such conditions an  $A_L$ -type phase-solubility diagram, with slope less than unity, would be observed, and the stability constant ( $K_{1:1}$ ) of the complex can be calculated from the slope and the intrinsic solubility ( $S_0$ ) of the drug in the aqueous complexation media (i.e., drug solubility when no cyclodextrin is present):

$$(2) \quad K_{1:1} = \text{Slope} / [S_0(1 - \text{Slope})]$$

The value of  $K_{1:1}$  is most often between 50 and 2000  $M^{-1}$  with a mean value of 129, 490 and 355  $M^{-1}$  for  $\alpha$ -,  $\beta$ - and  $\gamma$ -cyclodextrin, respectively [12,29-31]. For 1:1 drug/cyclodextrin complexes the complexation efficiency (CE) can be calculated from the slope of the phase-solubility diagram:

$$(3) \quad CE = [D/CD]/[CD] = S_0 \cdot K_{1:1} = \text{Slope} / (1 - \text{Slope})$$

When selecting cyclodextrin or complexation conditions during formulation work it can frequently be more convenient to compare the CE than  $K_{1:1}$  values. The most common stoichiometry of higher order drug/cyclodextrin complexes is the 1:2 drug/cyclodextrin complex resulting in  $A_P$ -type phase-solubility diagram. Consecutive complexation is assumed where the 1:2 complex is formed when one



**Figure 3. Results from a kinetic study of the effect of cyclodextrin on drug stability.** Degradation of chlorambucil in 2-hydroxypropyl- $\beta$ -cyclodextrin (HP $\beta$ CD) solution (10 mM NaH<sub>2</sub>PO<sub>4</sub>/NaOH buffer, pH 7.5, 30 °C).

additional cyclodextrin molecule forms a complex with an existing 1:1 complex [24]:



The stoichiometry of the system can be probed by curve fitting of the diagram with a quadratic model:

$$(5) \quad S_{tot} = S_0 + K_{1:1}S_0[CD] + K_{1:1}K_{1:2}S_0[CD]^2$$

Here [CD] represents the concentration of free cyclodextrin, but it is customary to plot the total amount of dissolved drug ( $S_{tot}$ ) against the total amount of cyclodextrin in solution ( $[CD]_{tot}$ ), assuming that the extent of complexation is low (i.e.,  $[CD] \sim [CD]_{tot}$ ). The value of  $K_{1:2}$  is frequently between 10 and 500 M<sup>-1</sup>, or significantly lower than that of  $K_{1:1}$ .

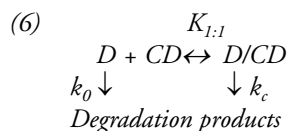
Various methods can be applied to prepare drug/cyclodextrin complexes, including the solution method, the co-precipitation method, neutralisation method, the slurry method, the kneading method, and the grinding method [23,32]. In most cases presence of at least some water is essential for successful complex formation. In solution, cyclodextrin complexes are usually prepared by addition of excess amount of drug to an aqueous cyclodextrin solution. The suspension formed is equilibrated at the desired temperature (which may require periods of up to 1 week) and then filtered or centrifuged to form clear drug/cyclodextrin complex solution. For preparation of solid complexes, the water is removed from the aqueous drug/cyclodextrin solution by evaporation (e.g., spray drying) or sublimation (e.g., lyophilisation).

For a variety of reasons, such as isotonicity of parenteral formulations and formulation bulk of solid dosage forms, it is important to include as little cyclodextrin as possible in a pharmaceutical formulation. Various methods have been applied to enhance the complexation efficacy [33]. These include addition of polymers to the complexation media [34], drug ionisation and salt formation [35,36], addition of hydroxy carboxylic acids to the complexation media [37], addition of volatile acids or bases to the complexation media [38], addition of organic salts [39], and addition of cosolvents [40]. However, even under the best conditions, cyclodextrin complexation will result in over fourfold increase in the formulation bulk of solid dosage forms [33]. Rao and Stella [31] have shown how the feasibility of using cyclodextrins in dosage forms can be calculated from few simple experiments.

### 2.3 Drug stability

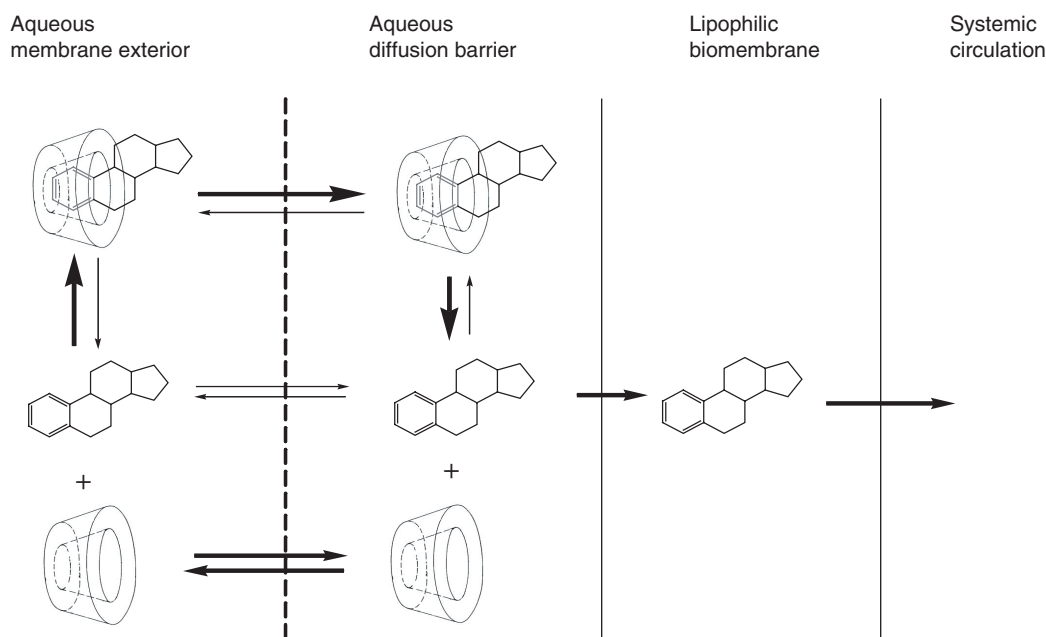
Stability issues can limit the feasibility of a pharmaceutical formulation. This is especially true for aqueous formulations of drugs that are prone to hydrolysis or oxidation. The reaction rates can be affected by inclusion of the drug, and especially inclusion of its chemically labile moiety, into the cyclodextrin cavity.

In cyclodextrin solutions the observed degradation rate for a chemically unstable compound, forming 1:1 complex, will be the weighted average of the degradation rates of the free drug and drug in cyclodextrin complex. In first-order and pseudo-first-order reactions, such as hydrolysis or oxidation, the stabilising (or catalytic) effect will depend on three parameters: the cyclodextrin concentration, the stability constant of the complex, and the degradation rate constant for the drug degradation within the cyclodextrin cavity ( $k_c$ ). These parameters can be determined by fitting degradation data obtained at several cyclodextrin concentrations to Equation 7:



$$(7) \quad k_{obs} = (k_0 + k_c K_{1:1}[CD]) / (1 + K_{1:1}[CD])$$

Where  $k_{obs}$  is observed rate constant for the reaction and  $k_0$  is the rate constant for the reaction in pure aqueous solution. An example of such kinetic study, of chlorambucil degradation in aqueous 2-hydroxypropyl- $\beta$ -cyclodextrin solution, is shown in Figure 3 [41]. Through nonlinear fitting of Equation 7, the values for  $K_{1:1}$  ( $3836 \pm 89$  M<sup>-1</sup>, mean  $\pm$  standard deviation),  $k_0$  and  $k_c$  can be obtained ( $k_0/k_c = 17$ ). From Figure 3 it can be seen that 50% of the maximum stabilising effect is obtained when the cyclodextrin concentration is equal to  $1/K_{1:1}$ . In many of the older publications the values of  $k_c$  and  $K_{1:1}$  were obtained by linear fitting to Equation 8 [42]:

**Table 3. The effect of cyclodextrin complexation on drug bioavailability after non-parenteral administration.**

FDA class*	Drug properties		RDS to drug absorption <sup>¶</sup>	Effect of cyclodextrin complexation
	<b>Aqueous solubility<sup>‡</sup></b>	<b>Permeability<sup>§</sup></b>		
I	Highly soluble	Highly permeable	(Good bioavailability)	Can decrease absorption
II	Poorly soluble	Highly permeable	Aqueous diffusion	Can enhance absorption
III	Highly soluble	Poorly permeable	Membrane permeation	Can decrease absorption
IV	Poorly soluble	Poorly permeable	Aqueous diffusion and membrane permeation	Can enhance absorption

\*FDA Biopharmaceutics Classification System of orally administered drugs. <sup>‡</sup>Intrinsic solubility of the drug in the aqueous membrane exterior. <sup>§</sup>Passive drug permeation through lipophilic biomembrane such as the gastrointestinal mucosa. <sup>¶</sup>RDS of drug delivery from the aqueous exterior into the body.

FDA: Food and Drug Association; RDS: Rate determining step.

$$(8) \quad 1/(k_0 - k_{obs}) = 1/(k_0 - k_e) + 1/K_{1:1} (k_0 - k_e) 1/[CD]$$

However, because the nonlinear method is less sensitive to experimental errors than the linear one, it frequently gives more accurate results [43,44]. Many studies have shown that stability of chemically labile drugs and compounds such as steroid esters [45], alkylating anticancer agents [46-49], prostaglandins [50-52], prodrugs [53,54] and various other drug compounds [9,42] can be improved through formulation with cyclodextrins. Most of these studies have been focused on drug stability in aqueous solutions, but cyclodextrins have also been shown to stabilise drugs in solid dosage forms [55,56]. The photochemistry of cyclodextrin inclusion complexes has been studied [57] and the effect of complexation on photostability of drugs has been investigated [58]. These studies have shown that cyclodextrin complexation can affect the light absorption properties of the inclusion compound and primary and secondary photochemical reactions, and that the effect can be either stabilising or destabilising. In

general, these effects are modest and may depend on the type of cyclodextrin used and on the experimental conditions, such as the irradiation wavelength. Finally, cyclodextrins can also increase physical stability of drugs. For example, evaporation of volatile compounds can be significantly reduced through complex formation [32], and cyclodextrins have been used to prevent aggregation and to reduce denaturation in peptide and protein formulations [59-61].

Cyclodextrins can sometimes have a destabilising effect on drugs through direct catalysis or, for example, by enhancing drug solubility in aqueous drug suspensions [9,42]. Frequently, the catalytic effect is associated with deprotonisation of the hydroxy groups located at the rim of the cyclodextrin cavity [62-64]. In this way cyclodextrins behave like carbohydrates and other polyhydric alcohols with adjacent hydroxy groups [63]. In this case the catalytic effect will mainly be observed under basic conditions and will increase with increasing pH.

## 2.4 Drug delivery through biological membranes

The chemical structure of cyclodextrins (i.e., the large number of hydrogen donors and acceptors), their molecular weight (i.e., > 970 Da) and their very low octanol/water partition coefficient (approximately  $\log P_{o/w}$  between -3 and 0.00 [65]) are all characteristics of compounds that do not readily permeate biological membranes [4,66]. In fact, experiments have shown that only negligible amounts of hydrophilic cyclodextrins and drug/cyclodextrin complexes are able to permeate lipophilic membranes such as skin and gastrointestinal mucosa [11,67]. Only the free form of the drug, which is in equilibrium with the drug/cyclodextrin complex, is capable of penetrating lipophilic membranes [68]. Cyclodextrins are able to extract lipophilic components from biomembranes such as stratum corneum [69,70], but both pre- and postapplication of hydrophilic cyclodextrins does not affect, for example, the skin barrier [71,72]. Cyclodextrins do not, in general, enhance permeability of hydrophilic water-soluble drugs through lipophilic biological membranes [73,74], and numerous studies have shown that excess cyclodextrin will reduce drug permeability through biological membranes [73]. The physicochemical properties of the drug (e.g., its solubility in water), the composition of the drug formulation (e.g., aqueous or non-aqueous) and physiological composition of the membrane barrier (e.g., presence of an aqueous diffusion layer), will determine whether cyclodextrins will enhance or hamper drug delivery through a biological membrane (Table 3). Most biological membrane barriers (or biomembranes) are lipophilic with an aqueous exterior, which forms a structured water layer at the membrane surface frequently referred to as unstirred diffusion layer. If drug permeation through the aqueous diffusion layer is the rate-limiting step of drug permeation through the barrier, cyclodextrins can frequently enhance the permeation. However, cyclodextrins are in most cases unable to enhance drug permeation through a lipophilic membrane barrier and excess cyclodextrin (more than is needed to dissolve the drug) will hamper drug permeation through the membrane. In other words, cyclodextrins will enhance drug delivery through aqueous diffusion-controlled barriers, but can hamper drug delivery through lipophilic membrane-controlled barriers [74]. However, there is one exception: lipophilic cyclodextrins, such as the methylated  $\beta$ -cyclodextrins, are able to permeate mucosa and are known to enhance drug delivery through biological membranes, such as through the nasal mucosa, by reducing barrier function of the membranes [75].

Cyclodextrins can, at least in theory, enhance drug bioavailability by stabilisation of drug molecules at the biomembrane surface. For example, cyclodextrins have been shown to prevent insulin aggregation and to enhance insulin stability at the nasal mucosa. It has been suggested that cyclodextrin-enhanced insulin bioavailability after nasal administration is partly due to this stabilising effect [76]. In general, drug stabilisation associated with cyclodextrin complexation plays only a very minor role when it comes to drug delivery through biological membranes.

It is their solubilising effect that is usually related to improved drug delivery. However, as cyclodextrins can both enhance and hamper drug delivery through biological membranes it is of utmost importance to optimise cyclodextrin-containing drug formulations with regard to drug delivery from the formulations [73]. Too much or too little cyclodextrin can result in less than optimum drug bioavailability.

## 3. Formulation with cyclodextrins

It is important for a pharmaceutical formulator to know the advantages and limitations of each excipient used during design of a product. Excipients are selected based on the physicochemical properties of the drug (e.g., solubility, stability etc.), type of delivery (e.g., tablet, parenteral solution etc.) and desired pharmacokinetics (e.g., instant release, sustained release etc.). The following is a brief overview of the use of cyclodextrins in various formulations, with the main emphasis on the effects of cyclodextrins on aqueous solubility and drug permeability through biological membranes. However, cyclodextrins are also able to increase the physical and chemical stability of drugs in the various formulations (i.e., increasing the shelf-life of the pharmaceutical product) as well as to reduce local drug irritation.

### 3.1 Oral drug delivery

Drug absorption from immediate-release tablets in the gastrointestinal tract consists of a series of rate processes including drug dissolution in the aqueous gastrointestinal fluids, permeation of the drug molecules from the intestinal fluid through an aqueous diffusion layer immediately adjacent to the mucosal surface, and permeation through the mucosa. The effect of cyclodextrins on oral drug absorption can be explained in the context of the Biopharmaceutics Classification System (Table 4) [77]. The Biopharmaceutics Classification System categorises drugs according to their aqueous solubility and ability to permeate the intestinal mucosa (Table 3). A given drug substance is considered 'highly soluble' when the highest dose strength is soluble in  $\leq 250$  ml water over a pH range of 1.0 – 7.5, and 'highly permeable' when the extent of oral absorption in humans is determined to be  $\geq 90\%$  of an administered dose (in solution). For an immediate-release tablet,  $\geq 85\%$  of the labelled amount of drug substance must dissolve within 30 min [2,4,301]. Class I drugs are relatively water soluble and their absolute bioavailability is  $\geq 90\%$ . These drugs permeate easily through the aqueous diffusion layer and possess sufficient lipophilicity to partition into and then permeate through the gastrointestinal mucosa. In general, hydrophilic cyclodextrins are not able to improve bioavailability of Class I drugs. However, cyclodextrin can be used to reduce local drug irritation and increase rate of drug absorption. Class II drugs have limited aqueous solubility, resulting in dissolution-rate limited oral absorption. However, once in solution these drugs permeate biological membranes relatively easily, resulting in  $\geq 90\%$  absolute bioavailability. Thus, low

**Table 4. Some examples of cyclodextrins in oral formulations, tested *in vivo* in humans and/or animals, and the effect of the cyclodextrin complexation on the absolute bioavailability compared to identical cyclodextrin-free formulation.**

Drug	Cyclodextrin	Formulation	Species	F <sub>rel</sub> <sup>*</sup>	Ref.
<b>Class I</b>					
Piroxicam	βCD	Tablet, capsule and oral suspension	Human, rat, rabbit	≤1.4	[128-131]
<b>Class II</b>					
Carbamazepine	DMβCD	Oral powder and solution, tablet	Rabbit, dog, rat	≤5.6	[132-136]
Digoxin	γCD	Tablet	Dog	5.4	[137]
Glibenclamide	βCD, SBEβCD	Capsule containing powder	Dog, rat	≤6.2	[138, 139]
Miconazole	HPβCD	Aqueous suspension	Rat	2.3	[140]
Phenytoin	E-βCD, GluβCD, MalβCD, SBEβCD, HPβCD	Suspension, capsule containing powder	Rat, Dog	≤5	[141-143]
Spironolactone	βCD, γCD, DMβCD, SBEβCD, HPβCD	Oral solution and powder	Rat, dog	≤3.6	[144-146]
Tolbutamide	βCD, HPβCD	Suspension, oral powder	Rabbit, dog	≤1.5	[147, 148]
α-Tocopheryl nicotinate	DMβCD	Capsule containing powder	Dog	~70	[149]
<b>Class III</b>					
Acyclovir	βCD	Oral suspension	Rat	1.1	[150]
Diphenhydramine HCl	DMβCD, HPβCD	Solution	Rat	≤0.9	[151]
<b>Class IV</b>					
Cyclosporin A	DMβCD	Oral suspension	Rat	4.7	[152, 153]

\*F<sub>rel</sub> (i.e., the AUC of the plasma concentration versus time profile when the cyclodextrin-containing formulation was given divided by the AUC for the formulation containing no cyclodextrin).

AUC: area-under-curve; βCD: β-Cyclodextrin; γCD: γ-Cyclodextrin; DMβCD: Dimethyl-β-cyclodextrin; E-βCD: β-Cyclodextrin epichlorohydrin polymer;

F<sub>rel</sub>: Relative bioavailability; GluβCD: Glucosyl-β-cyclodextrin; HPβCD: 2-Hydroxypropyl-β-cyclodextrin; MalβCD: Maltosyl-β-cyclodextrin;

SBEβCD: Sulfobutylether-β-cyclodextrin sodium salt.

aqueous solubility hampers their dissolution rate. The drug permeation through the aqueous diffusion layer adjacent to the mucosal surface will also be slow due to their low aqueous solubility. Water-soluble cyclodextrin complexes of these drugs will enhance their diffusion to the mucosal surface leading to enhanced oral bioavailability. Class III drugs are water soluble, but do not easily permeate biological membranes due to, for example, their size and/or extent of hydration. Consequently, formation of hydrophilic drug/cyclodextrin complexes will not enhance their oral bioavailability, but will, if anything, reduce the ability of dissolved drug molecules to partition from the aqueous exterior into the gastrointestinal mucosa. Class IV drugs are water insoluble and do not readily permeate lipophilic biological membranes. These can, for example, be water-insoluble zwitterions or relatively large lipophilic molecules. Hydrophilic water-insoluble compounds such as zwitterions do not readily form cyclodextrin complexes and, thus, hydrophilic cyclodextrins are not likely to improve their oral bioavailability. However, cyclodextrins are able to improve aqueous solubility of some large lipophilic molecules leading to increased drug availability at the mucosal surface. This will frequently lead to increased oral bioavailability.

### 3.2 Sublingual drug delivery

Sublingual drug delivery is one of the most efficient ways to bypass hepatic first-pass metabolism [78]. However, in order to enter into the systemic circulation the drug must dissolve in the saliva. Due to the small volume of saliva in the mouth, the therapeutic dose has to be relatively small and usually dissolution enhancers must be applied.

In sublingual formulations the complexation of poorly water-soluble drugs with cyclodextrins has been shown to increase the bioavailability of various lipophilic drugs. For example, 2-hydroxypropyl-β-cyclodextrin has been shown to increase the bioavailability of 17β-oestradiol [79,80], androstenediol [81], clomipramine [82] and danazol [83]. In the case of lipophilic compounds, the aqueous solubility and dissolution rate of a drug is usually the rate-limiting step for drug absorption and, after dissolution, the drug readily penetrates through mucosal membranes. Thus, in most studies, the increased bioavailability achieved by cyclodextrins is likely to be due to increased aqueous solubility and drug dissolution rate. However, interactions between cyclodextrins and sublingual mucosa (i.e., cyclodextrins acting as conventional penetration enhancers) cannot be excluded.

From a toxicological point of view the use of cyclodextrins in sublingual formulations is closely related to other forms of oral administration. The large hydrophilic cyclodextrin molecules do not permeate across the sublingual mucosa and, thus, they are eventually swallowed. However, there are some basic differences between sublingual administration and oral administration of cyclodextrin-containing formulations. As discussed earlier, the drug must be released from the inclusion complex before it can be absorbed. This can be a problem for sublingual applications due to the small volume of aqueous saliva and the relatively short residence time. The dissolved drug is removed from the buccal area within few minutes after administration; therefore not allowing enough time for the drug to be released from the cyclodextrin complex.

One limitation in the use of cyclodextrins in sublingual administration is the effect of cyclodextrins on formulation bulk. For example, in the development of sublingual formulations of  $\Delta^9$ -tetrahydrocannabinol (THC) for the treatment of various medical conditions, the complexation of THC with 2-hydroxypropyl- $\beta$ -cyclodextrin and randomly methylated  $\beta$ -cyclodextrin was studied. The result showed that the estimated therapeutic dose (1 mg) of THC could form a water-soluble complex with 400 mg of 2-hydroxypropyl- $\beta$ -cyclodextrin, but formulation bulk of 400 mg is considered too large for sublingual administration. However, the complexation efficiency of randomly methylated  $\beta$ -cyclodextrin with THC was much higher and, thus, the same amount of THC (1 mg) could form a soluble complex with 25 mg of randomly methylated  $\beta$ -cyclodextrin, which made the development of sublingual THC formulations possible [201]. Results from *in vivo* absorption studies showed that sublingual administration of randomly methylated  $\beta$ -cyclodextrin containing THC formulation increases the bioavailability of THC compared with oral administration [84].

### 3.3 Nasal drug delivery

The nasal route is another effective way to bypass the hepatic first-pass metabolism [85]. Because of the good permeability properties of nasal mucosa, the nasal route has also been studied as a possible administration route for systemic delivery of peptides. However, in order to enter the systemic circulation the drug has to dissolve in the aqueous nasal fluids. In nasal formulations, cyclodextrins are normally used to increase the aqueous solubility of lipophilic drugs. However, lipophilic cyclodextrins can also interact with biological membranes, acting as penetration enhancers, especially in nasal delivery of peptides [75]. Numerous studies have demonstrated that methylated cyclodextrins in particular are efficient absorption enhancers, and this is one reason why they are the most commonly studied cyclodextrins in nasal drug delivery [86]. The first cyclodextrin-based nasal formulations contained steroidal hormones and peptides [76,87-89]. The results were very promising and the most effective cyclodextrins, methylated cyclodextrin derivatives, increased, for example, the bioavailability of progesterone threefold compared with suspension of the same

compound [90]. For example, nasal bioavailability of insulin in rats was increased from about 0 to 100% by including methylated cyclodextrins in the formulation [91]. However, much lower insulin bioavailability after nasal administration was later observed in human studies and it is well known that there are large interspecies differences associated with nasal drug delivery [86]. Recently, promising results from nasal delivery of dihydroergotamine [92], midazolam [93], acyclovir [94] and heparins [95] have been reported. Only insignificant amounts of cyclodextrins are absorbed from the nasal cavity. Most cyclodextrins are removed from the cavity by the nasal mucociliary system, which transports cyclodextrins to the oesophagus and ultimately into the gastrointestinal tract. The local toxicity of cyclodextrins after nasal administration is very low. The acute histological effects of the lipophilic methylated cyclodextrins, for example, were close to physiological saline when studied in rats [96]. In addition, the local toxicity of dimethyl- $\beta$ -cyclodextrin, indicated by ciliary beat frequency, has been shown to be very mild compared with other absorption-enhancing agents and preservatives (e.g., benzalkonium chloride) used in nasal formulations [97].

The amount of cyclodextrins that can be used in nasal formulations is limited by the fact that only 25 – 150  $\mu$ l of liquid can be sprayed into each nostril [85]. The oestradiol nasal spray Aerodiol® (Servier) represents the successful use of cyclodextrins in nasal applications; each spray delivers 70  $\mu$ l of solution, which contains 150  $\mu$ g of oestradiol dissolved in aqueous randomly methylated  $\beta$ -cyclodextrin solution.

### 3.4 Pulmonary drug delivery

Pulmonary administration of drugs is usually intended for local treatment of diseases (i.e., to treat asthma, chronic obstructive pulmonary disease or other lung diseases) [98]. However, pulmonary drug delivery is also an attractive route for systemic drug delivery. Drug degradation in the gastrointestinal tract and first-pass metabolism can be circumvented by administration via the lungs. Lungs have a large surface area, the blood flow to the lungs is high and the enzymatic activity in the lungs is relatively low, all of which generate good conditions for effective drug absorption. However, pulmonary drug delivery can be limited by low aqueous solubility and slow drug dissolution. Insoluble particles are removed from the lungs by the mucociliary clearance in the upper airways and by macrophages in the alveoli [98]. Cyclodextrins can be of value in pulmonary delivery by increasing the solubility, stability and dissolution rate of water-insoluble and chemically unstable drugs. This can lead to decreased clearance, increased drug absorption and faster onset of drug action. Furthermore, by forming drug/cyclodextrin complexes, a liquid drug can be converted to a solid form, two incompatible drugs can be mixed in a dry powder formulation, bad smells and/or tastes can be reduced, and local drug irritation in the lungs can be reduced.

Cyclodextrins are more readily absorbed from the lungs than from the gastrointestinal tract and this limits the



**Table 5. Current marketed drug formulations [154] compared with cyclodextrin formulations (unpublished results and [155]).**

	Commercial product	Cyclodextrin formulation
<b>Diazepam i.v. solution (Roche)</b>		
Diazepam	5 mg/ml	5 mg/ml
Propylene glycol	40%	
Ethyl alcohol	10%	
Sodium benzoate/benzoic acid	5%	
Benzyl alcohol	1.5%	
Water for injection	~ 43%	~ 93%
2-Hydroxypropyl- $\beta$ -cyclodextrin		6%
Sodium chloride		0.6%
<b>Phenytoin i.v. solution (Parke-Davis)</b>		
Phenytoin sodium	50 mg/ml	50 mg/ml
Propylene glycol	40%	
Alcohol	10%	
Sodium hydroxide	adjust pH to 12	adjust pH to 11
Water for injection	~ 43%	~ 75%
2-Hydroxypropyl- $\beta$ -cyclodextrin		20%

number of cyclodextrins that can be included in pulmonary formulations but, in general, cyclodextrins that are considered safe for parenteral administration are also considered safe for pulmonary administration [11,99]. Among the cyclodextrins used in pharmaceutical products,  $\gamma$ -cyclodextrin, 2-hydroxypropyl- $\beta$ -cyclodextrin and sulfobutylether  $\beta$ -cyclodextrin are considered to be the safest for parenteral administration. However, the number of studies dealing with the toxicity or local effects of cyclodextrins on lung cells is very limited. A 72-h treatment with aqueous 0.1 – 1% 2-hydroxypropyl- $\beta$ -cyclodextrin solutions did not have any significant effect on growth of CRL7272 human lung cells *in vitro* [100]. The toxicity of natural cyclodextrins, 2-hydroxypropyl- $\beta$ -cyclodextrin and randomly methylated  $\beta$ -cyclodextrin was also studied recently with Calu-3 pulmonary epithelial cell line *in vitro* [101]. The results showed that cyclodextrins are well tolerable in Calu-3 cells and decreased the cell viability only at the high cyclodextrin concentrations.

The number of studies dealing with pulmonary applications of cyclodextrins is also very limited. Studies have been performed using premeasured dry powder inhalers, which emit the dose from a pierced blister or capsule [102]. The respirable fraction of salbutamol from Diskhaler® (GlaxoSmithKline) has been increased by complexation with  $\gamma$ -cyclodextrin and dimethyl- $\beta$ -cyclodextrin [104], and the respirable fraction of beclomethasone dipropionate from Microhaler® has been increased by

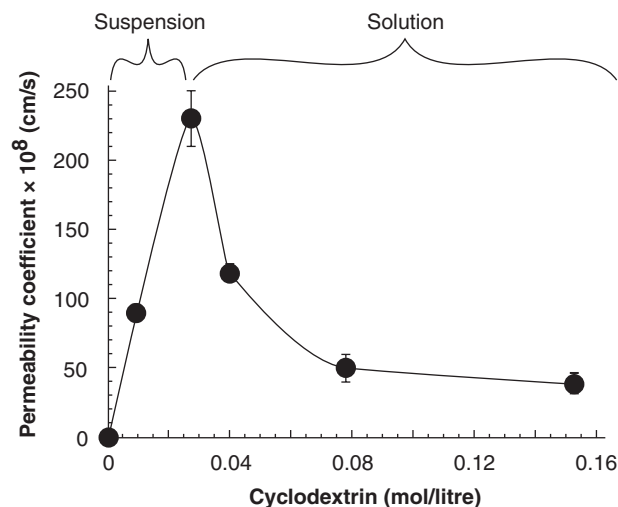
2-hydroxypropyl- $\beta$ -cyclodextrin complexation [104]. Furthermore, the absorption of intratracheally administered drugs has been shown to increase in the presence of various cyclodextrins [105-107]. A recent study with budesonide also showed that cyclodextrin complexes could be used in an inhalation powder without lowering the pulmonary deposition of the drug [108].

### 3.5 Injectable formulations

Injectable formulations of lipophilic water-insoluble drugs frequently consist of mixtures of water, organic cosolvents and surfactants. Limitations in using organic solvents in injectable formulations include possible drug precipitation, pain, inflammation and haemolysis on injection [109]. Sometimes it is possible to alleviate these side effects by designing a water-soluble prodrug of the lipophilic water-insoluble drug. However, a prodrug will change the pharmacokinetics of the parent drug. For example, due to the gradual metabolism of the prodrug to form the active drug, the onset time of the drug (i.e., the time required for the drug to reach minimum effective plasma concentration) will be delayed [110,111]. Organic solvents and surfactants can be replaced by isotonic aqueous cyclodextrin solutions (Table 5). Numerous studies have shown that unlike prodrugs these aqueous cyclodextrin vehicles containing the active drug will in general not alter the intrinsic pharmacokinetics of a drug [10,12]. On parenteral administration, especially after intravenous injection, the drug is both rapidly and quantitatively released from the cyclodextrin complex upon dilution, competitive replacement, and binding of drug molecules to plasma proteins and tissue [113]. However, because cyclodextrins are rapidly eliminated in the urine cyclodextrins can increase renal clearance of lipophilic water-insoluble drugs [112]. Finally, the hydrophilic cyclodextrin derivatives, such as 2-hydroxypropyl- $\beta$ -cyclodextrin and sulfobutylether  $\beta$ -cyclodextrin, are relatively non-toxic compared with organic solvents and surfactant formulations. Furthermore, as they have a minimal effect on the intrinsic pharmacokinetics of drugs, cyclodextrin-containing formulations are increasingly being used during *in vitro* and *in vivo* screening of new pharmacologically active compounds.

### 3.6 Ophthalmic drug delivery

In ophthalmology local drug administration in the form of topically applied low viscosity aqueous eye drop solutions is preferred. The outermost layer of the eye cornea is a lipophilic epithelium and, thus, drugs must be somewhat lipophilic to be able to permeate through the cornea into the eye. However, attached to microvilli at the corneal surface is an aqueous layer of about 8  $\mu$ m thick and, thus, topically applied drugs must be water soluble to be able to penetrate this aqueous diffusion barrier to reach the corneal surface [113]. In addition, only one eye drop, or 0.03 – 0.05 ml, can be applied to the eye, which means that in aqueous eye drop solution the drug dose must be soluble in < 0.05 ml of the aqueous formulation. The average tear volume is only 7  $\mu$ l and any excess liquid is rapidly spilled onto the skin or drained through the nasolacrimal duct



**Figure 4.** The permeability coefficient of arachidonylethanolamide through the isolated cornea of pigmented rabbits as a function of 2-hydroxypropyl- $\beta$ -cyclodextrin concentration. The vehicle consisted of 0.5 mg/ml of the drug in suspension or solution containing from 0.000 to 0.155 mol/litre cyclodextrin. Approx. 0.03 mol/litre cyclodextrin was needed to dissolve 0.5 mg/ml of the drug. Modified from reference [156].

into the nose. In addition, continuous secretion of tear fluid limits the contact time of topically applied drugs with the eye surface. Consequently, < 5% of a topically applied drug is absorbed into the eye [114,115]. Through cyclodextrin solubilisation it is possible to increase the dose-to-solubility ratio, making it possible to apply drugs topically that previously could only be given by systemic delivery [115,116]. For example, acetazolamide is a carbonic anhydrase inhibitor that is used to treat glaucoma with oral daily dose as high as 1000 mg. The aqueous solubility of acetazolamide in pure water is 0.7 mg/ml, but in 20% (w/v) aqueous 2-hydroxypropyl- $\beta$ -cyclodextrin solution it is 7 mg/ml. Addition of water-soluble polymers to the aqueous cyclodextrin solution increases the solubility even further. Thus, it is possible to obtain topically effective acetazolamide eye drop solution through cyclodextrin solubilisation of the drug [117].

Cyclodextrin solubilisation of the drug will increase the amount of dissolved drug at the lipophilic membrane surface (i.e., enhance drug delivery through the aqueous diffusion barrier), but excess cyclodextrin (i.e., more than is needed to dissolve the drug) will decrease the ability of the drug molecules to partition into the lipophilic barrier. Thus, excess cyclodextrin can result in decreased drug delivery through the cornea (Figure 4). Cyclodextrins have also been used to reduce ophthalmic drug irritation and to increase chemical stability of drugs in aqueous ophthalmic formulations [115,118].

### 3.7 Dermal drug delivery

It is generally believed that the main barrier to drug absorption, into and through the skin, is the outermost layer of the

skin: stratum corneum. Penetration enhancers used in dermal drug formulations, such as fatty acids and alcohols, penetrate into stratum corneum and temporarily decrease its barrier properties. However, only negligible amounts of topically applied hydrophilic cyclodextrins are able to penetrate into the stratum corneum and they have negligible effect on its barrier properties [10,67,68,73]. Numerous studies have shown that excess cyclodextrins do, like in the case of ophthalmic drug delivery (Figure 4), decrease drug delivery through excised skin [73]. Cyclodextrins enhance drug delivery through aqueous diffusion layers (i.e., aqueous diffusion barriers), but not through lipophilic barriers such as the stratum corneum. If the drug release is from an aqueous-based vehicle or if an aqueous diffusion layer at the outer surface of the skin is a rate-determining factor in dermal drug delivery, then cyclodextrins can act as penetration enhancers (Table 3). However, if drug penetration through the lipophilic stratum corneum is the main rate-determining factor then cyclodextrins are unable to enhance the delivery [74]. It appears that cyclodextrins do enhance hydrocortisone delivery from an unstirred aqueous donor phase through hairless mouse skin, but have no effect on hydrocortisone delivery from a well-stirred donor phase [74,119]. In general, cyclodextrins do not enhance drug delivery from non-aqueous vehicles [68]. For example, it has been shown that both  $\beta$ -cyclodextrin and 2-hydroxypropyl- $\beta$ -cyclodextrin reduce the amount of hydrocortisone released from non-aqueous petrolatum-based vehicles and from w/o cream (water-in-oil emulsion), but enhance the release from both o/w cream (oil-in-water emulsion) and a hydrogel formulation [120]. When applied to excised human skin, cyclodextrins enhanced hydrocortisone delivery from the o/w cream, but reduced the delivery from the non-aqueous petrolatum-based vehicle [121]. As cyclodextrins enhance dermal delivery of drugs by increasing the amount of drug at the surface of the stratum corneum, and conventional penetration enhancers enhance drug delivery by decreasing the barrier function of the stratum corneum, it is possible to obtain an additive effect by combining the two types of enhancers [122,123]. For example, in one study the effects of both 2-hydroxypropyl- $\beta$ -cyclodextrin and a conventional penetration enhancer (i.e., glycerol monoether extract) on transdermal delivery of testosterone, from o/w cream through hairless mouse skin, was investigated *in vitro* [124]. An ~ 60% increase in the testosterone flux was obtained when cyclodextrin was added to the cream, about a 40% increase occurred when the extract was added to the cream, but about an 80% increase in the flux was observed when both cyclodextrin and the extract were added to the cream.

Cyclodextrins have also been used to reduce permeability of compounds into skin. For example, addition of an excess of 2-hydroxypropyl- $\beta$ -cyclodextrin to a vehicle containing the UV-absorbing compound oxybenzone (a common sunscreen) (more than needed to solubilising the compound) reduced significantly transdermal permeation of the compound [125]. In addition, studies have indicated that complexation of the

sunscreen enhances its photoprotective effects by preventing permeation of the sunscreen into skin [126].

#### 4. Expert opinion and conclusion

Recent advances in drug development, such as high-throughput screening, have increased the number of drug candidates whose clinical usefulness is hampered by their insolubility in water. Furthermore, the usefulness of a number of drugs and drug candidates is hampered by their chemical or physical instability, or local irritation after administration. Cyclodextrins can alleviate many of these undesirable drug properties. Worldwide there are currently about 30 different cyclodextrin-containing pharmaceutical products on the market in about 14 different types of formulations, including different types of tablets (i.e., conventional, chewing and sublingual tablets), oral capsules, parenteral solutions, suppositories, nasal sprays, eye drop solutions and dermal products. In these products cyclodextrins are used to replace organic solvents in parenteral and topical formulations, to enhance oral bioavailability of Class II and some Class IV drugs, to reduce gastrointestinal irritation and to increase dermal availability of drugs. Furthermore, studies in both humans and animals have shown that cyclodextrins can be used to improve drug delivery from almost any type of drug formulation, but not all. The

outcome of a cyclodextrin formulation is highly dependent on the physicochemical properties of the drug being formulated. In addition, the pharmaceutical formulator has to possess a good knowledge of the physicochemical properties of cyclodextrins and their complexes to be able to apply this new technology successfully in drug delivery. Addition of cyclodextrins to existing formulations, without further optimisation, will seldom result in acceptable outcome.

We still lack deeper knowledge of the forces involved in the complex formation. Recent studies have shown that cyclodextrins form both inclusion and non-inclusion complexes, and that those complexes coexist in aqueous solutions. It has also been shown that cyclodextrins form aggregates in aqueous solutions and that those aggregates are able to act as solubilisers in a micellar-like fashion. However, we do not know the exact structures of these non-inclusion complexes and aggregates, nor do we know how they influence drug delivery from cyclodextrin-containing drug formulations. Novel cyclodextrin derivatives for site-specific drug delivery and gene delivery are being synthesised and tested in animals, as well as derivatives in which cyclodextrins are being used as pro-moieties in prodrugs intended for colon drug delivery. In addition, pharmacologically active cyclodextrin derivatives have recently been designed and shown to be clinically effective drugs. Thus, functionality of cyclodextrins will increase rapidly in the coming years.

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## MINIREVIEW

# Self-Association of Cyclodextrins and Cyclodextrin Complexes

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**ABSTRACT:** Cyclodextrins are useful functional excipients, which are being used in an ever-increasing way to camouflage undesirable pharmaceutical characteristics, especially poor aqueous solubility. It has generally been assumed that the mechanism whereby cyclodextrins exert their effects, especially their augmentation of solubility, is via the formation of noncovalent, dynamic inclusion complexes. This is a model, which regards drug–cyclodextrin interactions as a discrete phenomenon and ignores the possible interaction of these complexes with one another. It is becoming increasingly apparent that such assumptions may not be universally applicable or all encompassing. Specifically, there is a growing body of evidence that supports the important contribution of non-inclusion-based aspects for drug solubilization by cyclodextrins including surfactant-like effects and molecular aggregation. This short review attempts to assess the available literature for areas in which such non-inclusion mechanisms are apparent and tries to interpret these in the context of a broader working theory as to how cyclodextrins exert their beneficial effects. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 93:1091–1099, 2004

**Keywords:** cyclodextrin; self-association; complexation; non-inclusion; aggregates; phase-solubility; solubilization

## INTRODUCTION

It is generally assumed that when drug forms a complex with cyclodextrin, the molecule, in part or in whole, is taken up into the somewhat lipophilic cyclodextrin central cavity. In other words, an inclusion complex of the drug and cyclodextrin is formed.<sup>1</sup> Frequently, the stoichiometry of inclusion complexes is determined by simply fitting the phase-solubility diagrams, that is, the effect of cyclodextrin concentration on total drug solubility, to the appropriate equation without further verification.<sup>2,3</sup> In other instances, the stoichiometry of complexation is verified through

Job's plots or nuclear magnetic resonance (NMR) studies, but these are typically performed in dilute solutions in contrast with phase-solubility studies which are based on investigations of saturated drug solutions. Theoretical computer modeling can also be used to assess steric interaction of complexation and have been used to predict the nature of drug–cyclodextrin interaction in either vacuum or ideal solutions. Although these theoretical approaches continue to improve, at present they tend to be highly oversimplified descriptions and ignore important aspects of the formation of cyclodextrin complexes and their structure, especially in concentrated (i.e., nonideal) solutions.

It is well known that both noncyclic oligosaccharides and polysaccharides are able to form non-inclusion complexes.<sup>4–7</sup> It has also been shown that cyclodextrins are, like noncyclic oligosaccharides,

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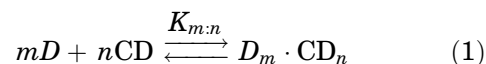
able to form non-inclusion complexes.<sup>8–13</sup> For example, Gabelica et al.<sup>8,9</sup> have reported that  $\alpha$ -cyclodextrin forms both inclusion and non-inclusion complexes with  $\alpha,\omega$ -dicarboxylic acids and that the two types of complexes coexist in aqueous solutions. By comparing  $\alpha$ -cyclodextrin complexes with those of maltohexaose, a linear analog of  $\alpha$ -cyclodextrin, the authors were able to show that the 1:1  $\alpha,\omega$ -dicarboxylic acid/ $\alpha$ -cyclodextrin complexes are mainly inclusion complexes whereas 2:1 complexes, formed by additional complex formation between a given acid and a 1:1 complex, are non-inclusion complexes. Even the 1:1 complexes were shown to consist of a mixture of inclusion and non-inclusion complexes. Other investigators have shown that acridine/dimethyl- $\beta$ -cyclodextrin 2:1 complex is formed when a 1:1 acridine/dimethyl- $\beta$ -cyclodextrin inclusion complex forms a non-inclusion complex with a second acridine molecule.<sup>13</sup> Furthermore, some 1:2 and 2:2 drug/cyclodextrin complexes have been shown to consist of a mixture of inclusion and non-inclusion complexes.<sup>14</sup> Having said this, in current complexation models, formation of non-inclusion cyclodextrin complexes is generally ignored.

Current stoichiometric models assume that once formed, the hydrated drug/cyclodextrin inclusion complexes are in an ideal solution in which individual complexes are independent of each other. However, several investigators have shown that both cyclodextrins and cyclodextrin complexes can self-associate to form aggregates of two or more cyclodextrin molecules or complexes.<sup>15–26</sup> In some cases, the aggregation results in opalescence of aqueous cyclodextrin solutions<sup>15</sup> but, in most cases, the diameter of the aggregates is much smaller than the wavelength of visible light and, consequently, only clear solutions are observed.<sup>18,19,24,25</sup> Studies have indicated that these water-soluble drug/cyclodextrin complex aggregates are effective solubilizers themselves, solubilizing lipophilic water-soluble drugs through non-inclusion complexation or through formation of micelle-like structures.<sup>23,24</sup> Current stoichiometric models do not account for formation of cyclodextrin aggregates and their effect on drug solubility.

## METHODS USED TO DETERMINE STABILITY CONSTANTS OF CYCLODEXTRIN COMPLEXES

In aqueous cyclodextrin solution, free drug molecules are in equilibrium with molecules bound to cyclodextrin molecules. The two most important

characteristics of the complexes are their stoichiometry and the numerical values of their stability constants. If  $m$  drug molecules ( $D$ ) associate with  $n$  cyclodextrin molecules (CD) to form a complex ( $D_m \cdot CD_n$ ), the following overall equilibrium is attained:



where  $K_{m:n}$  is the stability constant of the drug/cyclodextrin complex. In general, the physicochemical properties of free drug molecules are different from those bound to the cyclodextrin molecules. Likewise, the physicochemical properties of free cyclodextrin molecules are different from those in the complex. In general, any methodology that can be used to observe these changes in additive physicochemical properties may be utilized to determine the stoichiometry of the complexes formed and the numerical values of their stability constants.<sup>3,27</sup> These include changes in solubility, changes in chemical reactivity, changes in UV/VIS absorbance, changes in fluorescence, NMR chemical shifts, changes in drug retention (e.g., in liquid chromatography), changes in  $pK_a$  values, potentiometric measurements, and effects on drug permeability through artificial membranes. Furthermore, because complexation will influence the physicochemical properties of the aqueous complexation media, methods that monitor these media changes can be applied to study the complexation. These include measurements of conductivity changes, determinations of freezing-point depression, viscosity measurements, and calorimetric titrations. The stoichiometry of the drug/cyclodextrin complexes and the numerical values of their stability constants should be independent of the methodology applied and sometimes that is the case. Frequently, however, these values will depend on the specific method used for the determination. In Table 1, two methods, a fluorometric procedure and potentiometric approach using ion selective electrode, are compared. The former method utilizes a constant total drug concentration whereas the latter utilizes a constant cyclodextrin concentration. Significant differences were observed, especially when multiple complexes were formed.<sup>28</sup> The stability constants of 1:1 complexes of 5-phenylbarbituric acid and chlorpromazine with  $\beta$ -cyclodextrin, obtained from three different publications, are shown in Table 2. Again, significant discrepancies are observed, even between values obtained from the same publication.

**Table 1.** Stability Constants of 1-Anilino-8-naphthalenesulfonate (1,8-ANS) and 2-(*p*-Toluidinyl)-6-naphthalenesulfonate (2,6-TNS) with  $\beta$ -Cyclodextrin Determined by Fluorescence Method and by Using Ion Selective Electrodes<sup>28,29</sup>

Method	1,8-ANS	2,6-TNS	
	$K_{1:1}$ ( $M^{-1}$ )	$K_{1:1}$ ( $M^{-1}$ )	$K_{1:2}$ ( $M^{-1}$ )
Fluorescence	$110 \pm 4$	$1980 \pm 84$	$600 \pm 96$
Ion selective electrode	$86.9 \pm 1.2$	$3737 \pm 6$	$149 \pm 2$

Itraconazole is a relatively large lipophilic molecule (MW 706). The aqueous solubility of the drug has been estimated to be about 1 ng/mL at neutral pH and about 4  $\mu$ g/mL at pH 1.<sup>33</sup> Studies have indicated that the drug forms 1:1, 1:2, and 1:3 drug/2-hydroxypropyl- $\beta$ -cyclodextrin complexes.<sup>33–35</sup> However, significant differences are observed between the determined stability constants (Table 3, p. 1094). An NMR study indicated that the triazole and triazolone rings of the drug molecule could be involved in the 1:2 complex formation.<sup>34</sup> Because of the very low aqueous drug solubility, the NMR study of the itraconazole/cyclodextrin complex was done in pure dimethyl sulfoxide- $d_6$ . The authors argue that because the dielectric constants of dimethyl sulfoxide and water are similar ( $\epsilon = 46.8$  and 80, respectively), the stoichiometry of the complex should be similar. Although the NMR studies, together with the shape of the itraconazole-cyclodextrin phase-solubility profile, indicate that 1:2 itraconazole/2-hydroxypropyl- $\beta$ -cyclodextrin complex is being formed, the results are inconclusive. In particular, the curvature of the observed  $A_p$ -isotherm is such that no linear portion can be defined.

**Table 2.** Stability Constants ( $K_{1:1}$ ) of 5-Phenylbarbituric Acid (PBA) and Chlorpromazine (CPZ) 1:1 Complexes with  $\beta$ -Cyclodextrin at 25°C

Method	$K_{1:1}$ ( $M^{-1}$ ) and Reference	
	PBA	CPZ
pH-metric curve fitting	1590 <sup>30</sup>	3260 <sup>30</sup>
Ultraviolet spectrophotometry	1650 <sup>31</sup>	12,000 <sup>32</sup>
Circular dichroism	2940 <sup>31</sup>	7900 <sup>32</sup>
High-performance liquid chromatography	1860 <sup>31</sup>	8310 <sup>32</sup>
Solubility	3300 <sup>31</sup>	—

The stability constants of 1:1 complexes for the un-ionized (the acidic) and the ionized (the basic) forms of ibuprofen and naproxen with 2-hydroxypropyl- $\beta$ -cyclodextrin are listed in Table 4 (p. 1094). As expected, the complexes of the un-ionized forms appear to have larger stability constants than the ionized, more hydrophilic, forms, but significant variation is observed which is difficult to explain. For example, the stability constant for ionized ibuprofen 1:1 complex with 2-hydroxypropyl- $\beta$ -cyclodextrin is  $1550 M^{-1}$  when determined by capillary electrophoresis,  $533 M^{-1}$  when determined by a fluorescence method, and only  $50 M^{-1}$  when determined by the phase-solubility method. Likewise, the stability constant for ionized naproxen 1:1 complex with the same cyclodextrin is  $540 M^{-1}$  when determined by a fluorescence method but it has a much lower value when determined by the phase-solubility method. The various values of the stability constants of diflunisal/cyclodextrin 1:1 complexes are shown in Table 5 (p. 1095).

This variation in the observed stability constants could be due to differences in experimental technique, including the concentration of dissolved drug, but under ideal conditions, the different methods should give identical results. Ionic strength can sometimes have variable effect on phase-solubility diagrams. However, it has been shown that ionic strength has negligible effect on the binding of neutral molecules and, most frequently, negligible or negative effect on the binding of ionizable molecules.<sup>24,48,49</sup>

## PHASE-SOLUBILITY DIAGRAMS

The stoichiometry of drug/cyclodextrin complexes and the numerical values of their stability constants are frequently obtained from phase-solubility diagrams, that is, plots of drug solubility versus cyclodextrin concentration.<sup>2,50</sup> The phase-solubility technique was developed by Higuchi<sup>2,51</sup> and it is based on research related to how complexes of different complexing agents such as caffeine, polyvinylpyrrolidone, and some aromatic acids affect the aqueous solubility of drugs. These reports refer to  $\pi$ -complexation, but the principle should also apply to monomolecular inclusion complexation. Linear phase-solubility diagrams (Higuchi's  $A_L$ -type) indicate that the complex is first order with respect to the complexing agent [ $n = 1$  in eq. (1)] and first or higher order with respect to the drug ( $m \geq 1$ ). In

**Table 3.** Stability Constants of Itraconazole/2-Hydroxypropyl- $\beta$ -cyclodextrin in Aqueous Solution at pH 2.0 and 25°C

Method and Reference	Solvent	$K_{1:1}$ (M <sup>-1</sup> )	$K_{1:2}$ (M <sup>-1</sup> )
Solubility <sup>35</sup>	Water	3350	90
Solubility <sup>33</sup>	Water	8930	27
Solubility <sup>34</sup>	10% (v/v) propylene glycol in water	120	240
Ultraviolet spectrophotometry <sup>34</sup>	10% (v/v) propylene glycol in water	245	10

this case, the apparent drug solubility ( $S_{\text{tot}}$ ) will be given by:

$$S_{\text{tot}} = S_0 + m[D_m \cdot \text{CD}] \quad (2)$$

where  $S_0$  is the inherent solubility of the drug in the aqueous complexation medium. If one drug molecule forms a water-soluble complex with one cyclodextrin molecule (i.e., 1:1 complex), then the slope of the linear phase-solubility diagram will be determined by the equation:

$$\text{Slope} = \frac{S_0 K_{1:1}}{S_0 K_{1:1} + 1} \quad (3)$$

where  $K_{1:1}$  is the stability constant for the complex. In this case, the slope is always less than unity. If a 2:1 drug/cyclodextrin complex is formed, then the slope of the linear phase-solubility diagram will be determined by the equation:

$$\text{Slope} = \frac{2S_0^2 K_{2:1}}{S_0^2 K_{2:1} + 1} \quad (4)$$

where  $K_{2:1}$  is the stability constant of the complex. In this case, the slope of the linear phase-solubility diagram is always less than two.

Positive deviation from linearity ( $A_P$ -type phase-solubility diagrams) suggests formation of a higher-order complex with respect to cyclodextrin. The stoichiometry of the system can be probed by curve fitting with a quadratic model. A good fit to this model could suggest formation of a 1:2 drug/cyclodextrin complex:

$$S_{\text{tot}} = S_0 + K_{1:1} S_0 [\text{CD}] + K_{1:1} K_{1:2} S_0 [\text{CD}]^2 \quad (5)$$

where [CD] represents the concentration of free cyclodextrin. A third-order model is suggestive of a 1:3 complex, etc.<sup>3</sup> Here, consecutive complexation is assumed where, for example, a 1:2 complex is formed when one additional cyclodextrin molecule forms a complex with an existing 1:1 complex. Again, it is important to remember that this technique does not indicate whether a given drug forms inclusion complex with cyclodextrin, but only how the cyclodextrin influences the drug

**Table 4.** Stability Constants of Several Nonsteroidal Antiinflammatory Drugs, Carboxylic Acids, Assuming 1:1 Complexes with 2-Hydroxypropyl- $\beta$ -cyclodextrin Determined in Aqueous Cyclodextrin Solutions

Drug	Form	pH	Temperature (°C)	Method	$K_{1:1}$ (M <sup>-1</sup> )	Reference
Ibuprofen (pK <sub>a</sub> 5.2)	Un-ionized	4.6	30	Solubility	1740	36
		6 <sup>a</sup>	25	Solubility	5400	37
	Ionized	7	20	Fluorescence	533	38
	Ionized	7.4	25	Capillary electrophoresis <sup>b</sup>	1550	39
	Ionized	7.5	30	Solubility	50	36
Naproxen (pK <sub>a</sub> 4.2)	Un-ionized	<sup>c</sup>	25	Solubility	1670	40
	Un-ionized	~1	35	Fluorescence	2600	41
	—	4.6	30	Solubility	1400	36
	Mainly ionized	5 <sup>a</sup>	25	Solubility	2580	42
	Mainly ionized	6 <sup>a</sup>	25	Solubility	2600	37
	Ionized	7.4	30	Solubility	21	36
	Ionized	<sup>c</sup>	25	Solubility	331	40
	Ionized	~9	35	Fluorescence	540	41

<sup>a</sup>Unbuffered solution.

<sup>b</sup>The constant was determined by Scatchard analysis.

<sup>c</sup>Calculated values based on measurements in a series of aqueous buffer solutions.



**Table 5.** Stability Constant of Sodium Diflunisal/Cyclodextrin 1:1 Complexes in Aqueous Solutions

Method	Cyclodextrin	Temperature (°C)	$K_{1:1}$ ( $M^{-1}$ )	Reference
UV spectrophotometry	HP $\beta$ CD	25	5070	43
$^{19}F$ -NMR	HP $\beta$ CD	25	2030 <sup>a</sup>	43
Dialysis	HP $\beta$ CD	24	3890	44
Microcalorimeter	HP $\beta$ CD	25	3390	44
Potentiometric	HP $\beta$ CD	25	5570	45,46
UV spectrophotometry	$\beta$ CD	25	181,000	47
Potentiometric	$\beta$ CD	25	78,300	46
UV spectrophotometry	$\gamma$ CD	25	55,000	47
Potentiometric	$\gamma$ CD	25	88,200	46

The  $pK_a$  of diflunisal is 3.0.

<sup>a</sup>For the C2' signal.

solubility. Phase-solubility studies are performed in aqueous solutions saturated with the drug where formation of higher-order complex aggregates is more likely than in diluted unsaturated solutions.

Although correlation is often found between phase-solubility diagrams and the stoichiometry of drug/cyclodextrin complexes determined by other means such as NMR, some discrepancies can be found in the literature. For example, the slopes of linear (i.e.,  $A_L$ -type) phase-solubility diagrams of the sodium salts of ibuprofen and diflunisal in aqueous pH 6.0 phosphate buffer solution containing 2-hydroxypropyl- $\beta$ -cyclodextrin are 1.2 and 1.3, respectively.<sup>24</sup> This indicates that the complexes formed are first order with respect to cyclodextrin but second or higher order with respect to the sodium salts of ibuprofen and diflunisal. However, all other studies (including NMR investigations, Job's plots, and molecular modeling) indicate that the complexes formed are first order with respect to both cyclodextrin and the drugs. In addition, it has been shown that both diflunisal/cyclodextrin and ibuprofen/cyclodextrin complexes possess solubilizing properties in and of themselves, most likely by solubilizing drugs through non-inclusion solubilization or solubilization by complex aggregates.<sup>23,24</sup>

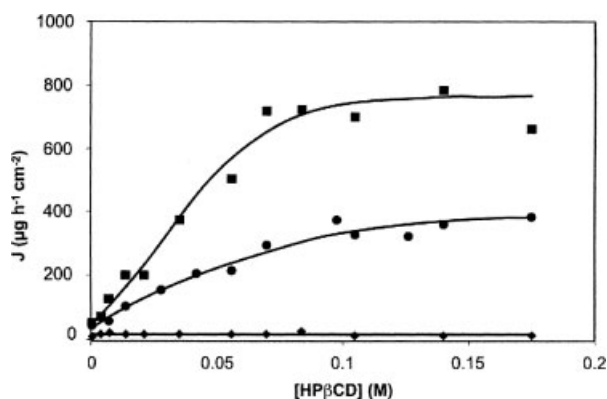
Because of electrostatic repulsions between the individual cyclodextrin molecules, sodium sulfobutylether  $\beta$ -cyclodextrin are thought to form exclusively 1:1 drug/cyclodextrin complexes.<sup>52</sup> However, the phase-solubility diagrams of sulfobutylether  $\beta$ -cyclodextrin are frequently of  $A_P$ -type. For example, the phase-solubility diagrams of cholesterol<sup>24</sup> and some pilocarpine prodrugs<sup>53</sup> in

aqueous sulfobutylether  $\beta$ -cyclodextrin solutions are of  $A_P$ -type, indicating formation of higher-order complexes with regard to cyclodextrin, but the profiles could not be fitted to the appropriate equations. It has been pointed out that  $A_P$ -type profiles have a strong resemblance to phase-solubility diagrams of lipophilic water-insoluble compounds in aqueous surfactant solutions. Thus, it has been suggested that 1:1 drug/cyclodextrin inclusion complexes form water-soluble non-inclusion complexes with additional drug molecules to give rise to  $A_P$ -type phase-solubility diagrams.<sup>24</sup> In fact, it has been shown that the solubility of cyclosporin A is about 33% higher in aqueous 2-hydroxypropyl- $\beta$ -cyclodextrin solutions that had previously been saturated with cholesterol compared with solutions that had not been saturated with cholesterol.<sup>54</sup> It is possible that the  $A_P$ -type phase-solubility diagrams observed for itraconazole in aqueous 2-hydroxypropyl- $\beta$ -cyclodextrin solutions may not be due to formation of higher-order complexes with respect to cyclodextrin but rather due to additional solubilization of itraconazole by the 1:1 complexes.

## CYCLODEXTRINS AND DRUG PERMEABILITY

Cyclodextrins are relatively large (MW from almost 1000 to more than 2000) hydrophilic molecules and thus cyclodextrins and their complexes do not efficiently permeate lipophilic biological membranes. Hydrophilic cyclodextrins enhance drug delivery through aqueous diffusion barriers but have no effect on drug permeation

through lipophilic membrane barriers.<sup>55,56</sup> The ability of cyclodextrins and their complexes to permeate semipermeable cellophane membranes depends on the molecular weight cutoff (MWCO) of the membranes.<sup>25,56</sup> Only relatively small drug molecules are able to permeate membranes with a MWCO of 500 but cyclodextrins and their complexes are able to permeate membranes with MWCO > 3000. Thus, it is possible to use semipermeable cellophane membranes to determine the stability constants of drug/cyclodextrin complexes.<sup>57</sup> The phase solubility of hydrocortisone in aqueous 2-hydroxypropyl- $\beta$ -cyclodextrin solutions is of  $A_L$ -type, indicating that a 1:1 drug/cyclodextrin complex is being formed and the stability constant of the 1:1 complex has been determined to be  $1400\text{ M}^{-1}$ .<sup>25</sup> The flux of hydrocortisone was determined from aqueous cyclodextrin solutions saturated with the drug through a series of membranes with MWCO from 500 to 14,000 (Fig. 1). Based on Fick's first law of passive diffusion, there should be a linear relationship between the hydrocortisone flux through the MWCO 500 membrane and the unbound hydrocortisone concentration in the donor solution, but in the case of the membranes with a MWCO of  $\geq 6000$ , the linear relationship should be between the total hydrocortisone concentration (i.e., bound and unbound to cyclodextrin) and the flux. The concentration of unbound hydrocortisone in the hydrocortisone-saturated cyclodextrin solutions is constant and equal to the inherent solubility of the drug. Thus, in accordance to Fick's first law,



**Figure 1.** The effect of 2-hydroxypropyl- $\beta$ -cyclodextrin (HP $\beta$ CD) concentration on the flux ( $J$ ) of hydrocortisone from aqueous solutions, saturated with hydrocortisone, through a semipermeable cellophane membrane; MWCO 500 (◆), MWCO 6000–8000 (●), MWCO 12,000–14,000 (■). From Ref.<sup>25</sup> with permission.

no increase in the flux through the MWCO 500 membrane was observed as the total concentration of hydrocortisone in the donor phase was increased (Fig. 1). However, the same solutions should have displayed a linear relationship when measured through the other two membranes with MWCO greater than the molecular weight of the hydrocortisone/cyclodextrin 1:1 complex. But the profiles show a strong negative deviation from linearity (Fig. 1). This deviation from linearity is thought to be due to self-association of cyclodextrin and cyclodextrin complexes to form aggregates that are unable to permeate the semipermeable membranes.<sup>22,24</sup> At increased concentrations, the hydrocortisone, cyclodextrin, and their complexes must form aggregates that have total molecular weight >6000–14,000. Thus, the actual stoichiometry of the hydrocortisone/cyclodextrin complex is much more complex than what can be anticipated from the simple hydrocortisone/cyclodextrin  $A_L$ -type phase-solubility diagram.

## CONCLUSIONS

In the classical cyclodextrin chemistry, it is assumed that when a drug molecule forms a complex with cyclodextrin, then some given lipophilic moiety of the drug molecule enters into the hydrophobic cyclodextrin cavity. In other words, that an inclusion complex of the cyclodextrin and drug is always formed. It is also assumed that once formed, the hydrated drug/cyclodextrin complexes are in an ideal solution in which individual complexes are independent of each other. Studies have shown that this is a significant oversimplification of a much more involved system. Cyclodextrins are able to form both inclusion and non-inclusion complexes. In addition, cyclodextrins and their complexes form water-soluble aggregates in aqueous solutions and these aggregates are able to solubilize lipophilic water-insoluble drugs through non-inclusion complexation or micelle-like structures.

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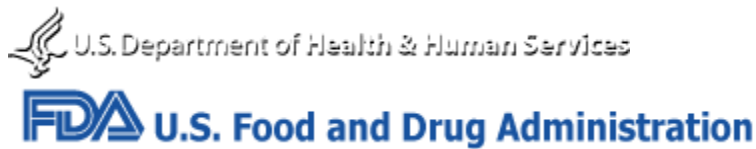
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<b>Orphan Designation:</b>	Treatment of the neurological manifestations of Niemann-Pick disease,type C.
<b>Orphan Designation Status:</b>	Designated
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<b>Generic Name:</b>	Miglustat
<b>Trade Name:</b>	Zavesca (R)
<b>Date Designated:</b>	11-12-2008
<b>Orphan Designation:</b>	Treatment of the neurological manifestations of Niemann-Pick disease,type C.
<b>Orphan Designation Status:</b>	Designated
<b>FDA Orphan Approval Status:</b>	Not FDA Approved for Orphan Indication
<b>Sponsor:</b>	Actelion Pharmaceuticals Ltd Innovation Centre CH-4123, Allschwil SWITZERLAND

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# Prevalence of Lysosomal Storage Disorders

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**L**YSOSOMAL STORAGE DISORDERS (LSDs) represent a group of at least 41 distinct genetic diseases, each one resulting from a deficiency of a particular lysosomal protein or, in a few cases, from nonlysosomal proteins, that are involved in lysosomal biogenesis. Most LSDs are inherited in an autosomal recessive manner, with the exception of Fabry disease and mucopolysaccharidosis (MPS) type II, which show X-linked recessive inheritance.

The number of LSDs is steadily increasing as new disorders are characterized biochemically and genetically. A deficiency of cathepsin K has recently been described that results in an LSD called *pyknodysostosis*.<sup>1</sup> In the last 2 years, infantile neuronal ceroid lipofuscinosis (NCL), also known as Batten disease, has been shown to result from a deficiency of palmityl protein thioesterase,<sup>2,3</sup> and classic late-infantile NCL has been shown to result from a deficiency of a carboxypeptidase.<sup>4</sup> Many LSDs have been classified into clinical subtypes (such as the Hurler-Scheie definition of MPS type I or the infantile-, juvenile-, and adult-onset forms of Pompe disease), but it is clear that most LSDs have a broad continuum of clinical severity and age of presentation.

With the advent of molecular biology and the characterization of many of the LSD genes, it is now recognized that the range of severity may in part be ascribed to different mutations within

**Context** Lysosomal storage disorders represent a group of at least 41 genetically distinct, biochemically related, inherited diseases. Individually, these disorders are considered rare, although high prevalence values have been reported in some populations. These disorders are devastating for individuals and their families and result in considerable use of resources from health care systems; however, the magnitude of the problem is not well defined. To date, no comprehensive study has been performed on the prevalence of these disorders as a group.

**Objective** To determine the prevalence of lysosomal storage disorders individually and as a group in the Australian population.

**Design** Retrospective case studies.

**Setting** Australia, from January 1, 1980, through December 31, 1996.

**Main Outcome Measure** Enzymatic diagnosis of a lysosomal storage disorder.

**Results** Twenty-seven different lysosomal storage disorders were diagnosed in 545 individuals. The prevalence ranged from 1 per 57 000 live births for Gaucher disease to 1 per 4.2 million live births for sialidosis. Eighteen of 27 disorders had more than 10 diagnosed cases. As a group of disorders, the combined prevalence was 1 per 7700 live births. There was no significant increase in the rate of either clinical diagnoses or prenatal diagnoses of lysosomal storage disorders during the study period.

**Conclusions** Individually, lysosomal storage disorders are rare genetic diseases. However, as a group, they are relatively common and represent an important health problem in Australia.

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the same gene. However, genotype-phenotype correlations do not always hold. In Gaucher disease, for example, there are sometimes substantial differences in the clinical manifestation of the disease between siblings and, in some instances, one sibling is severely affected while another is virtually free of disease.<sup>5</sup> Other factors, including genetic background and environmental factors, presumably play a role in disease progression.

Although each LSD results from mutations in a different gene and consequent deficiency of enzyme activity or protein function, all LSDs share a common biochemical characteristic in that the disorder results in an accumulation of normally degraded substrates within lysosomes. The particular substrates stored and the site(s) of storage vary, although

the substrate type is used to group LSDs into broad categories, including MPSs, lipidoses, glycogenoses, and oligosaccharidoses.<sup>6</sup> These categories show many clinical similarities within groups as well as significant similarities between groups. Common features of many LSDs include bone abnormalities, organomegaly, central nervous system dysfunction, and coarse hair and facies.

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There have been a number of reports on the prevalence of particular disorders in select populations. Of note is the level of Gaucher disease and Tay-Sachs disease in the Ashkenazi Jewish population, reported to be 1 per 855 and 1 per 3900, respectively.<sup>5,7</sup> Prevalences as high as 1 per 18 500 for aspartylglucosaminuria in the Finnish population<sup>8</sup> and 1 per 24 000 for MPS type III in the Netherlands<sup>9</sup> have also been reported. In addition, there have been a number of

limited studies on the prevalence of some LSDs in different countries.<sup>10-15</sup> However, in general, these studies have not been comprehensive and have not covered all LSDs. As the development of therapies for this group of disorders proceeds and the possibilities for neonatal screening are explored, it becomes important to obtain accurate values for prevalence of the disorders. These data will be required to accurately assess the cost of these disorders to public health

care systems and will be a key factor in the adoption of screening and treatment programs for LSDs.

In this article, we present a summary of all LSD diagnoses in Australia for the period 1980 through 1996.

## METHODS

In this study, patients diagnosed as having an LSD have a reduced level of 1 or more lysosomal proteins, which leads to the storage of substrate within their ly-

**Table 1.** Diagnosis of Lysosomal Storage Disorders in Australia\*

Disorder	Protein Deficiency	No. of Diagnoses†			Incidence in Thousands‡	Prevalence in Thousands§	Carrier Frequency
		Postnatal	Prenatal	Total			
Aspartylglucosaminuria	Aspartylglucosaminidase	2	0	2	2111	2111	726
Cystinosis	Cystine transporter	15	7	22	281	192	219
Fabry disease	α-Galactosidase	36	0	36	117	117	117 000¶
Gaucher disease	β-Glucocereamidase	71	3	74	59	57	119
G <sub>M1</sub> gangliosidosis	β-Galactosidase	10	1	11	422	384	310
Krabbe disease	Galactocereamidase	21	9	30	201	141	188
α-Mannosidosis	α-Mannosidase	4	0	4	1056	1056	514
Metachromatic leukodystrophy	Galactose-3-sulfatase	35	11	46	121	92	152
MPS type I (Hurler-Scheie)	α-Iduronidase	38	10	48	111	88	148
MPS type II (Hunter)	Iduronate-2-sulfatase	26	5	31	162	136	136 000¶
MPS type III-A (Sanfilippo A)	Glucosamine-N-sulfatase	33	4	37	128	114	169
MPS type III-B (Sanfilippo B)	α-N-Acetylglucosaminidase	18	2	20	235	211	230
MPS type III-C (Sanfilippo C)	Acetylcoenzyme A:α-glucosaminide-N-acetyltransferase	3	0	3	1407	1407	593
MPS type III-D (Sanfilippo D)	N-Acetylglucosamine-6-sulfatase	4	0	4	1056	1056	514
MPS type IV-A (Morquio A)	N-Acetylgalactosamine-6-sulfatase	21	4	25	201	169	206
MPS type VI (Maroteaux-Lamy)	N-Acetylgalactosamine-4-sulfatase	17	1	18	248	235	242
MPS type VII (Sly)	β-Glucuronidase	2	0	2	2111	2111	726
Mucopolidosis type II/III	Phosphotransferase	10	3	13	422	325	285
Multiple sulfatase deficiency	Multiple sulfatase factor, all sulfatases	3	0	3	1407	1407	593
Niemann-Pick disease type A/B	Shingomyelinase	16	1	17	264	248	249
Niemann-Pick disease type C	NPC1	20	0	20	211	211	230
Pompe disease	α-Glucosidase	21	8	29	201	146	191
Sandhoff disease	β-Hexosaminidase, β subunit	10	1	11	422	384	310
Sialic acid storage disease	Sialic acid transporter	7	1	8	603	528	363
Sialidosis	Neuraminidase	1	0	1	4222	4222	1027
Tay-Sachs disease	β-Hexosaminidase, α subunit	19	2	21	222	201	224
Wolman disease	Acid lipase	6	2	8	704	528	363
<b>Total/Combined</b>		<b>470</b>	<b>75</b>	<b>545</b>	<b>9.0</b>	<b>7.7</b>	<b>47</b>

\*MPS indicates mucopolysaccharidosis.

†Data are number of diagnoses made in Australia during 1980-1996.

‡Incidence was calculated by dividing the number of postnatal diagnoses by the number of births during the study period.

§Prevalence was calculated by dividing the number of postnatal plus prenatal diagnoses by the number of births during the study period.

||Carrier frequency was calculated by dividing the prevalence value by 4 and finding the square root.

¶Carrier frequency for X-linked disorders was assumed to be equal to the prevalence values because the incidence of carrier births should equal the prevalence of affected births for these disorders.

somes and results in the development of clinical problems and a subsequent reduction in their quality of life.

Retrospective data on the enzymatic diagnosis of LSDs, both from patient referrals and prenatal diagnoses for the period January 1, 1980, through December 31, 1996, were collected from the National Referral Laboratory, Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, Australia, and from the Division of Chemical Pathology, Royal Brisbane Hospitals, Brisbane, Australia. All diagnoses were performed at these 2 centers and this represents all enzymatic analyses performed in Australia during this period. No data were collected on the diagnosis of pyknodysostosis, glycogen-storage disease type II-B, or the various forms of Batten disease, which are currently not diagnosed enzymatically in Australia.

Data on the number of births in Australia were collected from the Australian Bureau of Statistics (Canberra). Data were compiled according to disorder, year of diagnosis, and age of patient (including prenatal diagnoses), and correlated with Australian birth rates for each year. Instances in which there were 2 or more affected siblings were identified.

Incidence rates were calculated by dividing the number of postnatal diagnoses by the number of births during the study period. Prevalence rates were calculated by dividing the number of postnatal plus prenatal diagnoses by the number of births during the study period. The total number with prenatal diagnoses who were not live-born were not included in the denominator because this figure was not accurately known and would not have made a significant difference to the prevalence figures. Carrier frequency was calculated by dividing the prevalence value by 4 and finding the square root. Carrier frequency for X-linked disorders was equal to the prevalence values because the incidence of carrier births should equal the prevalence of affected births for these disorders.

## RESULTS

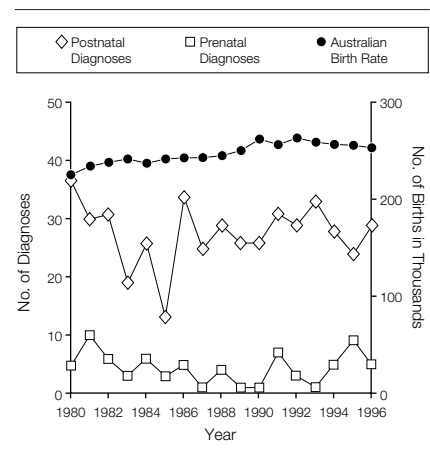
For the period January 1980 through December 1996, there were 470 LSD-

affected individuals diagnosed in the Australian population. In addition, there were 75 positive LSD prenatal diagnoses for affected fetuses, yielding a total of 545 diagnoses (TABLE 1). There was no significant increase in the rate of either clinical diagnoses or prenatal diagnoses during the study period (FIGURE). These diagnoses represent 27 different LSDs, whereas there were 10 LSDs for which there were no diagnoses in Australia during this period (TABLE 2). The prevalence of these disorders ranged from 1 per 57 000 for Gaucher disease to 1 per 4.2 million for sialidosis. The prevalence of LSDs as a group was calculated to be 1 per 7700 live births. When prenatal diagnoses were not considered, the incidence for LSDs was 1 per 9000 live births. Mucopolysaccharidosis, a particularly well-defined subgroup of LSDs, had a combined prevalence of 1 per 22 500 and represented 35% of all LSDs. Carrier frequencies were calculated from the prevalence values and ranged from 1 per 119 for Gaucher disease to 1 per 1027 for sialidosis (Table 1). Comparison of the number of LSD diagnoses in the different states of Australia (TABLE 3) indicates similar prevalence values in all major population centers. The exceptions, Australian Capital Territory, Northern Territory, and Tasmania, are all low-population areas that use genetic services in neighboring states and, as such,

would have had some patients recorded in those states.

We determined the median age of the patients at diagnosis for each LSD and report these together with the low and high values for each disorder (TABLE 4). Although for some disorders, the number of diagnoses was not high enough to make these data statistically significant, it still gives an indication of the range of ages at which these disorders can present. Most disorders (18/27) had more than 10 diagnoses in this period. These data demonstrate that certain disorders, in particular Fabry disease, can pre-

**Figure.** Postnatal and Prenatal Lysosomal Storage Disorder Diagnoses Made in Australia From 1980 Through 1996



**Table 2.** Disorders Not Detected Enzymatically in the Australian Population

Disorder	Protein Deficiency
Cobalamin deficiency type F	Cobalamin transporter
Farber disease	Acid ceramidase
Fucosidosis	$\alpha$ -Fucosidase
Galactosialidosis	Neuraminidase, $\beta$ -galactosidase, protective protein
Gaucher disease (variant)	Saposin C
Glycogen storage disease II-B*†	Unknown
$\beta$ -Mannosidosis	$\beta$ -Mannosidase
Metachromatic leukodystrophy (variant)	Saposin B
Mucopolysaccharidosis type IV-B (Morquio B)	$\beta$ -Galactosidase
Neuronal ceroid lipofuscinosis (infantile)*	Palmitoyl protein thioesterase
Neuronal ceroid lipofuscinosis (late infantile)*	Carboxypeptidase
Pyknodysostosis*†	Cathepsin K
Schindler disease‡	$\alpha$ -N-Acetylgalactosaminidase
Tay-Sachs disease type AB	$\beta$ -Hexosaminidase activator protein

\*Currently not screened for enzymatically in Australia.

†Prevalence levels are extremely low.

‡Screening in Australia commenced in 1995 (prevalence <1 per 500 000).



sent relatively late in life, with a mean age at diagnosis of 28.6 years, although for some individuals diagnosis was made in the first year of life. Clinicians should note the wide range of the clinical spectrum presenting in these disorders. In some LSDs, including Krabbe disease, MPS type I, Pompe disease, and Sandhoff disease, the median age at diagnosis was younger than 1 year, although the range of ages in each disorder still reflected a considerable variation in the clinical spectrum.

Of the 470 clinical diagnoses, there were 79 individuals who had 1 or more affected siblings. There were 4 families with twins, 9 families with an index case who in full knowledge had 1 or more additional affected children, and 26 families who had had 2 affected children before the first had an LSD diagnosed. In 9 of these 26 families, the affected individuals were adults (older than 18 years) when the disease was diagnosed.

## COMMENT

The prevalence values for individual LSDs clearly define these as rare genetic disorders. Gaucher disease was the most common, with a prevalence of 1 per 57 000 births. However, when taken as a group, LSDs are far more common, with a prevalence of 1 per 7700 births. Each year in Australia there are, on average, 28 LSD diagnoses made, with an additional 4 to 5 prenatal diagnoses. Although exact national figures for the number of MPS referrals are unavailable, in South Australia, 150 to 250 urine screening tests for MPS are performed each year to diagnose MPS, on average, in 1 patient. White-cell enzymology, which is performed for most other LSDs, is performed on 400 to 450 patients per year nationally, resulting in an average of 18 diagnoses. These estimates suggest considerable overlap between clinical features of LSDs and other conditions, but may also indicate the presence of additional as yet undefined LSDs. Although prenatal diagnosis is possible for most LSDs, no practical prenatal screening tests are available for any LSD.

The life expectancy of a patient with an LSD depends on the particular dis-

**Table 3.** Prevalence of Lysosomal Storage Disorders by State

State	No. of Births*	No. of Diagnoses†	Prevalence‡	95% Confidence Limits
Australian Capital Territory	80 211	2	40 106	ND§
New South Wales	1 450 878	207	7009	6554, 7533
Northern Territory	57 221	3	19 074	ND§
Queensland	719 534	78	9225	8287, 10 403
South Australia	332 210	44	7550	6561, 8890
Tasmania	116 842	9	12 982	9737, 19 472
Victoria	1 059 294	145	7305	6745, 7967
Western Australia	403 840	57	7085	6256, 8167
<b>Total Australia</b>	<b>4 222 323</b>	<b>545</b>	<b>7747</b>	<b>7429-8094</b>

\*Data are number of births recorded in each state during the period 1980-1996.

†Data are number of diagnoses (postnatal and prenatal) of all lysosomal storage disorders from each state during 1980-1996.

‡Prevalence was calculated by dividing the number of postnatal plus prenatal diagnoses by the number of births during the study period.

§ND indicates 95% confidence limits were not determined (number of diagnoses was insufficient to calculate meaningful confidence limit).

**Table 4.** Age at Diagnosis of Patients With Lysosomal Storage Disorders\*

Diagnosis	No. of Diagnoses†	Median Age (Range), y‡
Aspartylglucosaminuria	2	8.0 (7.0-9.0)
Cystinosis	14	1.0 (0.1-14.2)
Fabry disease	32	28.6 (0.0-55.7)
Gaucher disease	66	9.5 (0.0-73.2)
G <sub>M1</sub> gangliosidosis	9	1.2 (0.4-15.2)
Krabbe disease	20	0.5 (0.0-5.5)
α-Mannosidosis	2	10.5 (3.9-17.1)
Metachromatic leukodystrophy	31	2.3 (0.0-30.0)
MPS type I (Hurler-Scheie)	35	1.0 (0.3-29.1)
MPS type II (Hunter)	23	2.8 (0.0-22.0)
MPS type III-A (Sanfilippo A)	31	3.5 (0.2-17.1)
MPS type III-B (Sanfilippo B)	17	3.5 (0.0-21.4)
MPS type III-C (Sanfilippo C)	3	10.7 (5.7-12.0)
MPS type III-D (Sanfilippo D)	4	3.1 (0.4-7.4)
MPS type IV-A (Morquio A)	20	3.4 (0.0-19.0)
MPS type VI (Maroteaux-Lamy)	16	1.4 (0.0-43.4)
MPS type VII (Sly)	1	0.0
Mucopolidosis type II/III	10	0.8 (0.0-24.8)
Multiple sulfatase deficiency	2	8.1 (7.3-8.8)
Niemann-Pick disease type A/B	14	22.8 (0.7-44.1)
Niemann-Pick disease type C	19	9.3 (0.1-37.7)
Pompe disease	20	0.5 (0.1-55.0)
Sandhoff disease	9	1.0 (0.8-1.9)
Sialic acid storage disease	7	0.4 (0.0-36.1)
Sialidosis	1	0.3
Tay-Sachs disease§	18	1.1 (0.8-50.5)
Wolman disease	6	0.6 (0.1-5.5)
<b>Total Lysosomal Storage Disorders</b>	<b>432</b>	<b>2.7 (0-73.2)</b>

\*MPS indicates mucopolysaccharidosis.

†Data are number of postnatal diagnoses made in each group for which age data were available.

‡In some instances, early diagnosis was facilitated by an older affected sibling as an index case. Age at diagnosis of 0.0 refers to individuals diagnosed in the first 2 weeks after birth.

§Includes variants of hexosaminidase A deficiency.

order, the severity, and the treatment available. In MPS, this can range from lethal fetal hydrops to an almost-normal life expectancy.<sup>16</sup> In most, if not all, disorders, there is a strong correlation among age at diagnosis, severity, and life expectancy. The difference between the median age (2.7 years) and average age (9.7 years) at diagnosis of an LSD in Australia reflects the relatively few adult patients who have an almost-normal life expectancy. Based on the average age at diagnosis of 9.7 years and an average of 28 affected individuals born each year, we estimate that there are currently about 270 individuals with an undiagnosed LSD and possibly up to twice that number of patients with a diagnosed LSD in Australia.

That there were only 2 centers involved in the enzymatic diagnosis of LSDs greatly facilitated the collection of data for this study; as a consequence, we have a high level of confidence that few diagnoses were missed. Our confidence is supported by the state-by-state breakdown of the prevalence values for the LSDs, with the 5 major population centers showing similar prevalence data. Had 1 or more states missed a significant number of cases, this would present as uneven prevalence values among states. In addition, there was no significant variation in the number of diagnoses made per year during the study period; this would suggest that the patient identification rate is constant and close to 100% for these disorders. It is possible, however, that there are some individuals at the less-severe end of the clinical spectrum of some disorders, particularly in the adult population, in whom an LSD was not diagnosed. Gaucher disease is likely in this group. Similarly, we observed that there was no increase in the rate of prenatal diagnoses for the period of the study; this again reflects the steady rate of diagnosis of these disorders.

To calculate incidence, prevalence, and carrier frequency values, we needed to make certain assumptions. We assumed that the rate of postnatal diagnosis was equivalent to the birth rate for each disorder. If the postnatal diagnosis was less than the birth rate, as a result of undiagnosed early death, then our es-

timates of incidence values would be low. This is unlikely because all unexpected child deaths in Australia result in post-mortem examinations, including histopathology studies. We also assumed that the parents of affected individuals were heterozygous for the disorders (with the exception of X-linked disorders). If this were not the case, then our estimates of carrier frequency would be low; however, homozygous parents, in what are predominantly childhood disorders, are not common and, as such, would have little effect on our carrier frequency estimates.

The Australian population is predominantly of British extraction, with a significant contribution from other European countries and, to a lesser extent, Asian countries. As such, this population would be comparable with that of most Anglo-Celtic countries. Therefore, these results could be extrapolated to the white non-Hispanic populations in the United States, Canada, and the United Kingdom. However, a higher proportion of Ashkenazi Jews in a community may increase the prevalence of Gaucher disease and Tay-Sachs disease.

Although no data are available on the ethnic background of those diagnosed as having an LSD, we see no evidence that the Ashkenazi Jewish population contributed significantly to the figures for either Gaucher disease or Tay-Sachs disease in Australia. The Ashkenazi Jewish population in Australia is estimated to be 105 000 and is concentrated in Victoria and New South Wales. Despite this, we see no increase in the prevalence of either Gaucher disease or Tay-Sachs disease in these states compared with other states in Australia. This may be the result of outbreeding from the Jewish community into the general population. A screening program for the detection of Tay-Sachs disease carriers in the Jewish community was commenced in 1994; however, this would have had only a minimal effect on this study, which covered the period 1980 to 1996.

The cost to the community, in particular the health care system, of individuals with LSDs is significant. We have calculated the medical costs for a patient with

severe MPS I who has not had a bone marrow transplant to be approximately Aust \$80 000 (US \$56 000) per year, based on hospital admissions, hospital procedures, and outpatient visits during a 2.5-year period. In addition, such a patient would require full-time nursing home care while attending a special school, further increasing the cost to the community. Bone marrow transplantation costs, on average, Aust \$41 000 (US \$29 000). Enzyme replacement therapy for Gaucher disease currently costs between Aust \$140 000 and \$250 000 (US \$98 000-\$175 000) per year, although the cost for enzyme replacement therapy should decrease as more efficient enzyme production systems are developed. Given that some of the affected individuals are at the less-severe end of the clinical spectrum, the total cost to the community for individuals with an LSD in Australia is thought to be in the tens of millions of dollars per year. Although it is useful to determine the cost of these disorders to the community, in particular to the health care system, this represents only a fraction of the real cost, in human terms, of these disorders.

There were 39 families with more than 1 affected child; this highlights the need for early diagnosis of these disorders because in most cases, there were 2 affected children born before the first was diagnosed as having an LSD. Early detection of LSDs, such as that possible in the neonatal screening programs for phenylketonuria and other genetic diseases, would provide the option for prenatal diagnosis for many more families carrying these disorders. In addition, early detection would maximize the efficacy of current and proposed therapies for LSDs. The efficacy of these therapies, particularly for those LSDs involving central nervous system and bone pathologies, will rely heavily on the early diagnosis and treatment of the disorder, before the onset of irreversible disease. A further consideration, critical to bone marrow transplant therapy, is that early diagnosis of the LSD will allow clinicians to take advantage of the window of opportunity presented by the naturally suppressed immune system of the neonate to maximize the chances of a successful engraftment. Early intervention has

the potential to reduce costs associated with LSDs. Studies into the development of neonate screening for LSDs are currently in progress.<sup>17</sup>

Recently, there have been several advances made in the understanding of NCLs, a group of at least 4 disorders classified by age at onset. Previously, these disorders were diagnosed histopathologically rather than enzymatically and, consequently, we have no incidence data available. However, NCLs are relatively

common: estimates of incidence levels range from a global incidence for all forms of 1 per 12 500<sup>18</sup> to 1 per 78 000 for all forms in Germany.<sup>19</sup> In Finland, where there is a particularly high level, incidence values of 1 per 13 000 for infantile and 1 per 21 000 for juvenile forms have been reported.<sup>20</sup> Clearly, NCLs are going to contribute importantly to the overall prevalence of LSDs. A rate of 1 per 50 000 births for NCLs would alter the prevalence of LSDs from 1 per 7700 to 1 per

6700. Further epidemiological studies are required for this group of disorders.

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Nonviolence is the answer to the crucial political and moral questions of our time; the need for man to overcome oppression and violence without resorting to oppression and violence.

Man must evolve for all human conflict a method which rejects revenge, aggression and retaliation. The foundation of such a method is love.

—Martin Luther King, Jr (1929-1968)

#14 <http://www.census.gov/population/www/popclockus.html>

According to the U.S. Bureau of the Census, the resident population of the United States, projected to 02/16/10 at 18:36 UTC (EST+5) is  
308,697,268

According to the U.S. Bureau of the Census, the resident population of the United States, projected to 02/26/10 at 17:23 UTC (EST+5) is

308,763,255

COMPONENT SETTINGS FOR FEBRUARY 2010

One birth every.....	7 seconds
One death every.....	11 seconds
One international migrant (net) every.....	34 seconds
Net gain of one person every.....	13 seconds

The U.S. population clock is based on the national population estimates. The U.S. Census Bureau produces national population estimates annually using the latest available data on births, deaths, and international migration. Each year, we recalibrate the population clock when we release the new set of population estimates. For more information on the methodology for producing national estimates, see <http://www.census.gov/popest/topics/methodology/2008-nat-meth.pdf> [PDF].

[Historical National Population Estimates \[PDF - 81K\]](#)

Documentation for these projections.

[PDF] or denotes a file in Adobe's Portable Document Format. To view the file, you will need the Adobe® Acrobat® Reader available **free** from Adobe. This symbol indicates a link to a non-government web site. Our linking to these sites does not constitute an endorsement of any products, services or the information found on them. Once you link to another site you are subject to the policies of the new site.

**APPENDIX 2**  
**Table of Authorities**  
**Use of HPBCD in the Treatment of Niemann-Pick Disease Type C**

Compassionate use INDs #: 104,114 Addison Hempel and 104,116 Cassidy Hempel

Trappsol™ hydroxy-propyl-beta-cyclodextrin DMF No. 74-145





DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville, MD 20857

IND 104,114

Children's Hospital & Research Center Oakland  
Attention: Caroline Hastings, MD  
Director, Fellowship Program  
747 52<sup>nd</sup> Street  
Oakland, CA 94609-1809

**IND ACKNOWLEDGEMENT**

**RECEIVED**

**DEC 08 2008**

Dear Dr. Hastings:

We acknowledge receipt of your Investigational New Drug Application (IND) submitted under section 505(i) of the Federal Food, Drug, and Cosmetic Act (FDCA). Please note the following identifying data:

**IND NUMBER ASSIGNED:** 104,114

**SPONSOR:** Caroline Hastings, MD

**PRODUCT NAMES:** Trappsol Brand of Endotoxin Controlled Hydroxyl-Propyl-Beta-Cyclodextrin (HPBCD)

**DATE OF SUBMISSION:** November 25, 2008

**DATE OF RECEIPT:** November 25, 2008

You may not initiate studies in humans until 30 days after the date of receipt shown above unless we notify you sooner that you may proceed. If, on or before December 25, 2008, we identify deficiencies in the IND that require correction before human studies begin or that require restriction of human studies, we will immediately notify you verbally or in writing that (1) clinical studies may not be initiated under this IND ("clinical hold") or (2) certain restrictions apply to clinical studies under this IND ("partial clinical hold"). If we place your human studies on clinical hold, you will be notified in writing of the reasons and the information necessary to correct the deficiencies. In the event of such notification, you must not initiate or you must restrict such studies until you have submitted information to correct the deficiencies, and we have subsequently notified you that the information you submitted is satisfactory.

It has not been our policy to object to a sponsor, upon receipt of this acknowledgement letter, either obtaining supplies of the investigational drug or shipping it to investigators listed in the IND. However, if the drug is shipped to investigators, they should be reminded that studies may

not begin under the IND until 30 days after the IND receipt date or later if the IND is placed on clinical hold.

As sponsor of this IND, you are responsible for compliance with the FDCA (21 U.S.C. §§ 301 et. seq.) as well as the implementing regulations [Title 21 of the Code of Federal Regulations (CFR)]. A searchable version of these regulations is available at <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm>. Your responsibilities include (1) reporting any unexpected fatal or life-threatening adverse experiences associated with use of the drug by telephone or fax no later than 7 calendar days after initial receipt of the information [21 CFR 312.32(c)(2)]; (2) reporting any serious, unexpected adverse experiences, as well as results from animal studies that suggest significant clinical risk, in writing to this Division and to all investigators within 15 calendar days after initial receipt of this information [21 CFR 312.32(c)(1)]; and (3) submitting annual progress reports within 60 days of the anniversary of the date that the IND went into effect (the date clinical studies were permitted to begin) [21 CFR 312.33]. You are also responsible for complying with the applicable provisions of sections 402(i) and (j) of the Public Health Service Act (PHS Act) (42 USC §§ 282 (i) and (j)), which was amended by Title VIII of the Food and Drug Administration Amendments Act of 2007 (FDAAA) (Public Law No. 110-85, 121 Stat. 904).

Title VIII of FDAAA amended the PHS Act by adding new section 402(j) (42 USC § 282(j)), which expanded the current database known as ClinicalTrials.gov to include mandatory registration and reporting of results for applicable clinical trials of human drugs (including biological products) and devices. FDAAA requires that, at the time of submission of an application under section 505 of the FDCA, the application must be accompanied by a certification that all applicable requirements of 42 USC § 282(j) have been met. Where available, such certification must include the appropriate National Clinical Trial (NCT) numbers. 42 USC § 282(j)(5)(B). You did not submit such certification when you submitted this IND. You may use Form FDA 3674, *Certification of Compliance, under 42 U.S.C. § 282(j)(5)(B), with Requirements of ClinicalTrials.gov Data Bank*, to comply with the certification requirement. The form may be found at <http://www.fda.gov/opacom/morechoices/fdaforms/default.html>.

In completing Form FDA 3674, you should review 42 USC § 282(j) to determine whether the requirements of FDAAA apply to any clinical trial(s) referenced in this application. Additional information regarding the certification form is available at: [http://internet-dev.fda.gov/cder/regulatory/FDAAA\\_certification.htm](http://internet-dev.fda.gov/cder/regulatory/FDAAA_certification.htm). Additional information regarding Title VIII of FDAAA is available at: <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-08-014.html>. Additional information on registering your clinical trials is available at the Protocol Registration System website <http://prsinfo.clinicaltrials.gov/>.

We remind you that you may not charge for this investigational drug without prior written approval of FDA.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58). If such studies have not been conducted in compliance with these regulations, provide a statement describing in detail all differences between the

practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. Information on environmental assessments is available in the guidance *Environmental Assessment of Human Drugs and Biologics*. This document is available at <http://www.fda.gov/cder/guidance/1730fnl.pdf>.

Cite the IND number listed above at the top of the first page of any communications concerning this application. Each submission to this IND must be provided in triplicate (original plus two copies). Please include three originals of all illustrations that do not reproduce well. Send all submissions, electronic or paper, including those sent by overnight mail or courier, to the following address:

Food and Drug Administration  
Center for Drug Evaluation and Research  
Division of Gastroenterology Products  
5901-B Ammendale Road  
Beltsville, MD 20705-1266

All regulatory documents submitted in paper should be three-hole punched on the left side of the page and bound. The left margin should be at least three-fourths of an inch to assure text is not obscured in the fastened area. Standard paper size (8-1/2 by 11 inches) should be used; however, it may occasionally be necessary to use individual pages larger than standard paper size. Non-standard, large pages should be folded and mounted to allow the page to be opened for review without disassembling the jacket and refolded without damage when the volume is shelved. Shipping unbound documents may result in the loss of portions of the submission or an unnecessary delay in processing which could have an adverse impact on the review of the submission. For additional information, see <http://www.fda.gov/cder/ddms/binders.htm>.

If you have any questions, call me, at (301) 796-1007.

Sincerely,

*{See appended electronic signature page}*

Cristi L. Stark, M.S.  
Regulatory Health Project Manager  
Division of Gastroenterology Products  
Office of Drug Evaluation III  
Center for Drug Evaluation and Research

Linked Applications

Sponsor Name

Drug Name

IND 104114

Caroline Hastings, M.D.

Trappsol Brand of Endoloxin Controlled  
Hydroxyl-Propyl-Beta-Cyclodextrin  
(HPBCD)

**This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.**

/s/

CRISTI L STARK

12/01/2008



DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration  
Rockville, MD 20857

IND 104,116

Children's Hospital & Research Center Oakland  
Attention: Caroline Hastings, MD  
Director, Fellowship Program  
747 52<sup>nd</sup> Street  
Oakland, CA 94609-1809

**IND ACKNOWLEDGEMENT**

RECEIVED

DEC 08 2008

Dear Dr. Hastings:

We acknowledge receipt of your Investigational New Drug Application (IND) submitted under section 505(i) of the Federal Food, Drug, and Cosmetic Act (FDCA). Please note the following identifying data:

**IND NUMBER ASSIGNED:** 104,116

**SPONSOR:** Caroline Hastings, MD

**PRODUCT NAMES:** Trappsol Brand of Endotoxin Controlled Hydroxyl-Propyl-Beta-Cyclodextrin (HPBCD)

**DATE OF SUBMISSION:** November 25, 2008

**DATE OF RECEIPT:** November 25, 2008

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Cristi L. Stark, M.S.  
Regulatory Health Project Manager  
Division of Gastroenterology Products  
Office of Drug Evaluation III  
Center for Drug Evaluation and Research

Linked Applications

Sponsor Name

Drug Name

IND 104116

Caroline Hastings, M.D.

Trappsol Brand of Endoxin Controlled  
Hydroxyl-Propyl-Beta-Cyclodextrin  
(HPBCD)

**This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.**

/s/

CRISTI L STARK

12/01/2008

## **APPENDIX 3**

### **Summary of Animal Pharmacology and Toxicology**

#### **Pharmacology & Toxicology**

##### **b) In Vitro Pharmacology**

The physiological importance of cholesterol in the cell plasma membrane has attracted increased attention in recent years. Consequently, the use of methods of controlled manipulation of membrane cholesterol content has also increased sharply, especially as a method of studying putative cholesterol-enriched cell membrane domains (rafts). The most common means of modifying the cholesterol content of cell membranes is the incubation of cells or model membranes with cyclodextrins, a family of compounds, which, due to the presence of relatively hydrophobic cavity, can be used to extract cholesterol from cell membranes. However, the mechanism of this activity of cyclodextrins is not completely established.

Abulrob et.al. (*J Neurochem.* 2005 Mar;92(6):1477-86) demonstrated the neuroprotective activity of some CD derivatives against oxygen-glucose deprivation (OGD), N-methyl-D-aspartic acid (NMDA) and glutamate in cortical neuronal cultures. Although all CDs complexed with NMDA or glutamate, only beta-, methylated beta- and sulfated beta-CDs displayed neuroprotective activity and lowered cellular cholesterol.

Monnaert et.a. (*JPET* 310:7445-751, 2004) using an in vitro model of the blood-brain-barrier examined various cyclodextrin derivatives for their toxicity and permeability. The authors concluded that some cyclodextrins cross the BBB slightly in normal conditions.

##### **c) In Vivo Pharmacology**

After a single 200mg/KG intravenous dose and rats and dogs <sup>14</sup>C-HPBCD was eliminated rapidly (more than 90% in 4 hours), almost completely as the intact compound and mostly by renal excretion. The plasma elimination half-life was 0.4 hours and rats and 0.8 hours and dogs. In both rats and dogs following intravenous administration, tissue distribution was limited: in rats are highest concentrations were in the kidney and lung and in dogs the highest concentration was in the kidney and liver. The total plasma clearance in all species tested are similar to that of insulin and clearance through the kidneys and is independent of dose administered and nearly equivalent to the glomerular filtration rate. Thus the elimination is dependent on renal function.

##### **d) Use in NPC models**

Niemann-Pick type C is a rare autosomal recessive lysosomal storage disease and is related to mutation of the NPC1 protein responsible for cholesterol trafficking between the late endosomal and lysosomal compartments to the cytosol of all cells. As a result, clinical manifestations are organ dysfunction and ultimately neurological decline secondary to neurodegeneration. A homozygous mutant mouse (NPC<sup>-/-</sup>) has been identified that manifests many of the same defects seen in humans. This model has been used to elucidate the various functioning of the NPC1 gene and to screen for compounds with potential therapeutic potential.

Liu et.al. (J. Lipid Res, 2008, vol 49, pp663-669) demonstrated that a single 4000 mg/kg s.c. injection of HPBCD at 7 days of age prolonged the life of NPC<sup>-/-</sup> mutant mice. The effect size in that study, however, was a modest 15%. More recently, Walkley's group at Albert Einstein report (Abstract, Lysosomal Disease Network Annual Meeting, February 18-20, 2009) that NPC<sup>-/-</sup> mice treated daily from day 7 with 4000 mg/kg HPBCD demonstrated a 3 week delay in onset of clinical symptoms and survived more than double that of untreated NPC<sup>-/-</sup> mice.

Camargo et.al. (Life Sci. 2001, 70(2):131-42) found a similar small effect of HPBCD. They concluded the slight effects of the HPBCDs on neurological symptoms may be partially due to their apparent non-permeation of the blood-brain barrier (BBB). Intrathecal delivery of HPBCD by an Alzet osmotic minipump did not improve its efficacy in ameliorating neurological symptoms.

#### **e) Animal toxicology**

For an excellent review see: Gould and Scott "2-Hydroxypropyl-beta-cyclodextrin: A toxicology review (Food and Chemical Toxicology 43 (2005) 1451-1459.

##### **i) Oral**

Acute toxicity testing has not determined an LD(50) for HPBCD. 5000mg/kg have been administered orally to rats and no mortality was observed. (1)

C14 labeled HPBCD has been administered orally to determine the metabolic fate of the HPBCD. Most of the label was excreted in the feces. 3-6% of the label was absorbed and some label appeared in the blood about five minutes after administration indicating some absorption from the stomach. About 3% of the label appeared in the urine and another 3.25% in the exhaled CO<sub>2</sub>. The HPBCD preparation did contain some propylene glycol that was also labeled. The amount of label absorbed corresponded to the amount of propylene glycol present.

Teratogenicity and embryotoxicity studies have been done in rats and rabbits at doses up to 5000 mg/Kg per day in rats and 1000mg/Kg per day in rabbits. No maternal toxicity, embryotoxicity or teratogenicity was found in rats. In rabbits,

no teratogenicity was observed, but slight maternal and embryotoxicity was observed at 1000 mg/Kg.

Chronic studies were done using both mice and rats. The study with mice was terminated after 104 weeks. The mice received 500mg/Kg HPBCD. No adverse histopathology was noted that could be attributed to HPBCD and the mice given HPBCD had a lower incidence of tumors than the controls. A two-year study was also done with rats with a dose of 500 mg/Kg per day. No effects were observed that were attributed to HPBCD.

---

## **ii) Parenteral**

In an acute study with cynomolgus monkeys, a dose of 10, 000 mg/Kg was not lethal

HPBCD is quickly cleared after parenteral administration. After a single intravenous administration of (14)C labeled HPBCD, a half-life in the plasma of 0.4 hours and 0.8 hours was found for rats and dogs respectively. A plasma clearance of 512 and 188 ml/kg/h was calculated for rats and dogs.

The subchronic toxicity studies with rats, no adverse effects were found in rats treated intravenously with 50 mg/Kg HPBCD. At 100 mg/Kg HPBCD. At 100 mg/Kg some minimal histological changes were found in the epithelial cells of the urinary bladder, kidney tubular cells and in the liver. At 400 mg/Kg, there was a decreased body weight and food consumption, increased water consumption, decreased hematocrit, hemoglobin and erythrocyte levels, increased creatinine, total bilirubin and aspartate and alanine aminotransferase levels. Some organ weights also increase. Most of these changes were reversible after one month except for slightly elevated aspartate and alanine aminotransferase levels and histological changes in the lung and urinary tract that were only partially reversible.

In subchronic toxicity studies, no adverse effects were found in rats treated intravenously with 50mg/Kg or in dogs receiving 100 mg/KG HPBCD. At 400 mg/Kg in dogs there were slight increases in serum alanine and aspartate aminotransferase and total bilirubin. Histological changes were found in the lung and epithelial cells of the urinary bladder and renal pelvis. All of the changes were reversed within a month after treatment except for incomplete reversibility of the swollen renal pelvis epithelium.

Teratogenicity and embryotoxicity studies have been done in rats and rabbits at doses up to 400mg/Kg per day. Slight maternal toxicity was observed in rats at 400mg/Kg but there were no primary adverse effects in the offspring. No adverse effects were observed in the rabbits.

### **iii) Dermal**

Dermal irritation studies were done with albino rabbits. No erythema, edema or other dermal effects were observed and the material was considered to be a nonirritant to the skin. A dermal sensitization study was done using guinea pigs. No irritation was detected upon the initial intradermal injection of HPBCD. Upon challenge of applying HPBCD to the surface of the skin, low incidences of very faint erythema were found in 30% of the animals at 48 hours and 10% of the animals at 72 hours.

### **iv) Mutagenicity**

HPBCD is nonmutagenic. Mutagenicity was tested using *S. typhimurium* and *E. coli* WP2 strains both with and without microsomal activation and found to be non-mutagenic. Testing with mammalian cell culture, with and without microsomal activation, also found HPBCD to be non-mutagenic.



## APPENDIX 4

### Trappsol® (HPBCD) Product Labeling



#### Cyclodextrin Technologies Development Inc.

*Consultants to the Cyclodextrin Industry Worldwide*  
 27317 NW 78<sup>th</sup> Avenue, High Springs, Florida 32643  
 PH: (386) 454-0807, FAX: (386) 454-8134  
 E-mail: [scripps@cyclodex.com](mailto:scripps@cyclodex.com), Web site: [www.cyclodex.com](http://www.cyclodex.com)

#### Certificate of Analysis

#### THPB-EC

Trappsol® Hydroxypropyl Beta Cyclodextrin  
 Endotoxin Controlled

Lot No. 0608H7L162P\*\*

<u>ASSAY</u>	<u>SPEC'S</u>	<u>RESULT</u>
Identification	Conforms (EP)	Conforms
Appearance of Solution	Clear and Colorless (EP)	Conforms
Conductivity	≤ 200µS/cm [EP (2.2.38)]	98 mhos/cm
Loss on Drying	≤ 10.0% (PML-701)	3.0%
Purity	Min 98% (HPLC)	> 99%
Molar Substitution (QLPND02)	.083 - 1.14 (NMR)	1.0*
Heavy Metals	≤ 20 ppm (EP)	< 20 ppm
Unmodified DCD	≤ 1.5% (HPLC)	< 0.1%
Propylene Glycol (QLC001)	≤ 2.5% (GC) (EP)	< 0.2%
Bacterial Endotoxin	≤ 10 IU/g [EP (2.6.14)]	< 1 IU/g
Total Viable Aerobic Count (CFU/g)	≤ 100	< 10
Total Fungi (CFU/g)	≤ 100 (PML-705)	< 10
Salmonella (CFU/g)	Negative (PML-706)	Negative
E-Coli (CFU/g)	Negative (PML-706)	Negative

\*Approx corresponds to 7.0 DS

\*\* Meets or exceeds EP requirements for Hydroxypropyl Betadex

Released for use:

Date: 6/12/08

## **APPENDIX 5**

### **Investigational New Drug Application**