

Intracisternal cyclodextrin prevents cerebellar dysfunction and Purkinje cell death in feline Niemann-Pick type C1 disease

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Niemann-Pick type C1 (NPC) disease is a lysosomal storage disease caused by mutations in the *NPC1* gene, leading to an increase in unesterified cholesterol and several sphingolipids, and resulting in hepatic disease and progressive neurological disease. We show that subcutaneous administration of the pharmaceutical excipient 2-hydroxypropyl- β -cyclodextrin (HP β CD) to cats with NPC disease ameliorated hepatic disease, but doses sufficient to reduce neurological disease resulted in pulmonary toxicity. However, direct administration of HP β CD into the cisterna magna of presymptomatic cats with NPC disease prevented the onset of cerebellar dysfunction for greater than a year and resulted in a reduction in Purkinje cell loss and near-normal concentrations of cholesterol and sphingolipids. Moreover, administration of intracisternal HP β CD to NPC cats with ongoing cerebellar dysfunction slowed disease progression, increased survival time, and decreased the accumulation of brain gangliosides. An increase in hearing threshold was identified as a potential adverse effect. These studies in a feline animal model have provided critical data on efficacy and safety of drug administration directly into the central nervous system that will be important for advancing HP β CD into clinical trials.

INTRODUCTION

Niemann-Pick type C (NPC) disease is a severe inherited disorder characterized by progressive cerebellar ataxia, dementia, and early death due to neurological disease (1–3). More than 350 disease-causing mutations have been identified in the *NPC1* gene and more than 25 in the *NPC2* gene. NPC1 and NPC2 proteins normally function in concert to facilitate egress of unesterified cholesterol and sphingolipids from the late endosomal/lysosomal compartment (2, 4, 5). Dysfunction of either protein results in lysosomal storage of unesterified cholesterol and multiple sphingolipids (6–10), along with impaired export of lipoprotein-derived cholesterol (11–15). Despite the identification of causative mutations and a partial understanding of the function of the NPC1 and NPC2 proteins, the disease pathogenesis is not well understood.

The juvenile form of NPC disease, which is the most common, presents with progressive learning disabilities and ataxia beginning at 6 to 15 years of age that is often preceded by hepatosplenomegaly. Vertical supranuclear gaze palsy, cataplexy, seizures, dysarthria, and dysphagia are also seen, with death commonly occurring in the first or second decade (2, 16). Neuropathological abnormalities include

widespread neuronal cytoplasmic vacuolization, neuronal loss most severely affecting Purkinje cells, neuroaxonal dystrophy, gliosis, and inflammation (3, 7, 9, 17, 18). Lysosomal storage of unesterified cholesterol in neurons can be demonstrated by histochemical methods (8), whereas sphingolipid accumulation, particularly of gangliosides GM2 and GM3, can be demonstrated by both immunocytochemistry and biochemistry. Miglustat, a small imino sugar that partially inhibits glucosylceramide synthase and the synthesis of all glucosylceramide-based glycosphingolipids, delays the onset of clinical signs in animal models of NPC disease (19, 20). Whereas miglustat has been approved in Europe for the treatment of NPC disease since 2009 and subsequently in more than 40 countries, its use for the treatment of NPC disease remains off-label in the United States (21–23). There are currently no U.S. Food and Drug Administration (FDA)-approved therapies for NPC disease.

The cholesterol-lowering agents cholestyramine, lovastatin, and nicotinic acid and a low-cholesterol diet are ineffective in altering the neurological course of NPC disease (24, 25). However, in 2001, Camargo *et al.* evaluated the therapeutic effect of 2-hydroxypropyl- β -cyclodextrin (HP β CD) in a mouse model of NPC disease (26). Structurally, HP β CD contains a hydrophilic exterior and a hydrophobic interior, allowing it to increase the solubility of poorly water-soluble compounds such as cholesterol. Notably, in vitro studies showed that millimolar concentrations of HP β CD efficiently and rapidly removed cholesterol from cultured cells (27–29). In vivo, intraperitoneal or subcutaneous (SC) administration of HP β CD to *Npc1*^{-/-} mice decreased unesterified cholesterol storage in liver and delayed onset of neurological disease, increased life span, increased Purkinje cell survival, and reduced cerebrocortical cholesterol and ganglioside accumulation (26, 30, 31).

Given that HP β CD does not readily cross the blood-brain barrier (32), its apparent efficacy in the treatment of the neurological aspects

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of NPC disease is unexpected. To determine if direct intrathecal (IT) injection would be even more efficacious, we turned to a feline model of NPC disease. Feline NPC disease results from a single missense mutation in the *NPC1* gene (p.C955S) that is evolutionarily conserved and found in a cysteine-rich region commonly mutated in patients (33). Disease progression in this naturally occurring model recapitulates both the neuropathological and biochemical abnormalities observed in human patients, with the closest parallels to the juvenile form of NPC disease (9, 20, 34). In contrast to the murine model, the feline model is large enough to permit repeated administration of HP β CD either SC or IT and repeated sampling of blood and cerebrospinal fluid (CSF) to evaluate mechanistic, pharmacologic, and toxicity issues. This model also allows for validation of biochemical markers of disease severity and therapeutic effects that are specific to central nervous system (CNS) disease (20, 35–40).

Here, we show that administration of HP β CD into the subarachnoid space at the cisterna magna of affected cats completely resolved the clinical neurological signs of disease and Purkinje cell loss up to at least 24 weeks of age (the median age when untreated NPC cats die), providing critical data on efficacy and safety of drug administration directly into the CNS in a large animal model (41).

RESULTS

Untreated NPC cats developed CNS and hepatic disease and survived for a mean of 21 weeks

Thirty-nine untreated NPC cats were evaluated (Table 1). Untreated NPC cats showed increased serum hepatic alanine aminotransferase

(ALT) activity and serum total cholesterol, decreased serum albumin and body weight, and progressive cerebellar ataxia and intention tremor compared to normal control cats (Table 2, Fig. 1, A to C, fig. S1, and video S1). Untreated NPC cats were euthanized when they were nonambulatory and no longer able to remain in sternal recumbency without support, which occurred at a mean age of 20.7 ± 5 weeks (range, 9 to 29 weeks). Liver histology and biochemistry showed severe and extensive vacuolization of hepatocyte and Kupffer cell cytoplasm accompanied by pronounced increases in unesterified cholesterol, sphingomyelin, bis(monoacylglycerol)phosphate, glucosylceramide, lactosylceramide, globotriaosylceramide, free sphingosine (Fig. 2), and GM3 ganglioside (fig. S2A). Examination of the brain revealed diffuse neuronal cytoplasmic vacuolization with intracellular cholesterol storage identified by filipin staining, abundant storage of gangliosides (GM2 and GM3) in several brain regions examined (neocortex, caudate nucleus, and cerebellum), and severe Purkinje cell loss (Fig. 3 and fig. S3). Biochemical analysis of cerebral gray matter in untreated NPC cats with end-stage disease (median, 25 weeks; range, 21 to 29 weeks; $n = 9$) revealed that GM2 and GM3 constituted $17.4 \pm 0.6\%$ and $20.4 \pm 1.5\%$ of the total gangliosides, respectively, compared to a normal proportion of $2.5 \pm 0.6\%$ for GM3 and $2.0 \pm 0.5\%$ for GM2 (mean \pm SD) (Fig. 4A). Other biochemical lipid abnormalities were a marked accumulation of lactosylceramide (Fig. 4B) and glucosylceramide (fig. S2B) and a threefold increase of free sphingosine (Fig. 4C). Studies in younger NPC cats (Fig. 4D) showed that the increase in GM2 and GM3 gangliosides preceded the onset of neurological dysfunction. At 4 weeks of age, GM2 already constituted 9.5% of the total gangliosides and increased to 14% at 11 weeks. The increase of GM3 started later (5.3% at 4 weeks) but reached a higher concentration with time. A similar

Table 1. Route of administration and dose of HP β CD administered to 11 groups of cats.

Genotype	Route of administration	Dose	Dosing interval	Animal numbers		Survival time mean and SD, or longest (weeks)
				Males	Females	
No mutant allele (normal control)	Untreated	Untreated	Untreated	26	24	>76*
NPC1	Untreated	Untreated	Untreated	22	17	20.7 ± 5.0
NPC1	SC	1000 mg/kg and 25 mg/kg (allopregnanolone)	7 days	5	1	21.8 ± 6.5
NPC1	SC	4000 mg/kg	7 days	0	2	31, 36 [†]
NPC1	SC	8000 mg/kg	7 days	3	2	35.2 ± 12.4
NPC1	IC	3.8 mg	14 days	1	2	49 [†]
NPC1	IC	7.5 mg	14 days	1	2	62 [†]
NPC1	IC	15 mg	14 days	2	1	66 [†]
NPC1	IC	30 mg	14 days	1	5	>76*
NPC1	IC	60 mg	14 days	2	1	>76*
NPC1	IC	120 mg	14 days	2	7	>76*
NPC1	IC, SC	120 mg, 1000 mg/kg	14 days, 7 days	5	3	>76*
NPC1	IC	120 mg postsymptomatic [‡]	14 days	4	4	43.5 ± 5.8

*No cats in this group died of NPC disease during the study period of 76 weeks. †Longest survival time of individual cats that died due to signs of NPC disease. Remaining cats in this group were euthanized at ~24 weeks of age to collect histological data.

‡Treatment first began at 16 weeks of age.

Table 2. Serum ALT activity and albumin and cholesterol concentrations in NPC cats. Mean \pm SD for serum ALT, albumin, and total cholesterol of 24-week-old cats. *P* values are provided for groups significantly different from untreated normal control cats (*) or significantly different from untreated NPC cats (\dagger). SD is not provided for 4000 mg/kg group, where *n* = 2.

	Untreated normal control	Untreated NPC	1000 mg/kg SC	4000 mg/kg SC	8000 mg/kg SC	30 mg IC	120 mg IC	120 mg IC and 1000 mg/kg SC
ALT (U/liter)	62.1 \pm 15.4	395.8 \pm 169 * <i>P</i> = 0.000002	128.1 \pm 127.6 \dagger <i>P</i> = 0.0003	37.5	77 \pm 66.9 \dagger <i>P</i> = 0.00001	295.3 \pm 107 * <i>P</i> = 0.003	223.8 \pm 87 * <i>P</i> = 0.003 \dagger <i>P</i> = 0.003	135 \pm 91 \dagger <i>P</i> = 0.00004
Albumin (g/dl)	2.9 \pm 0.3	2.6 \pm 0.3 * <i>P</i> = 0.00035	2.8 \pm 0.2	3.0	3.3 \pm 0.38 * <i>P</i> = 0.002 \dagger <i>P</i> = 0.000002	2.5 \pm 0.01 * <i>P</i> = 0.00002	2.5 \pm 0.2 * <i>P</i> = 0.00006	3.0 \pm 0.3 \dagger <i>P</i> = 0.008
Cholesterol (mg/dl)	120.3 \pm 23	240.3 \pm 72 * <i>P</i> = 0.000001	189.9 \pm 48.5 * <i>P</i> = 0.004 \dagger <i>P</i> = 0.04	131	166 \pm 58 \dagger <i>P</i> = 0.04	191.2 \pm 37.3 * <i>P</i> = 0.004 \dagger <i>P</i> = 0.04	180.6 \pm 22.4 * <i>P</i> = 0.0001 \dagger <i>P</i> = 0.004	166 \pm 34.6* * <i>P</i> = 0.007 * \dagger <i>P</i> = 0.002

developmental pattern has been described in NPC mouse models (42). Finally, no clinical evidence of pulmonary disease was evident in untreated NPC cats, although lung histology showed foamy macrophages within alveolar spaces and septa (Fig. 5A). For comparison, histological, biochemical, and clinical data obtained in normal control cats are reported in Table 2 and Figs. 2 to 5.

SC HP β CD ameliorated hepatic disease and improved CNS disease only at doses with negative pulmonary consequences

Before assessing the potential effects of SC administration of HP β CD on NPC cats, we first determined serum and CSF concentrations in 24-week-old normal control cats 60 min after administration of a single dose of HP β CD. The achieved serum concentrations were 546, 2570, and 3485 μ g/ml, after administration of 1000, 4000, and 8000 mg/kg, respectively. CSF concentrations of HP β CD were not detectable in cats receiving 1000 mg/kg and were 19 and 21.1 μ g/ml in cats receiving 4000 and 8000 mg/kg, respectively. A full pharmacokinetic (PK) study was not performed.

To test therapeutic efficacy in NPC cats, HP β CD was repeatedly administered SC at one of three doses [1000 mg/kg with allopregnanolone (25 mg/kg), 4000 mg/kg, or 8000 mg/kg] every 7 days to NPC cats beginning at 3 weeks of age (Table 1). All three groups showed improvements in body weight (fig. S1) and in serum ALT, albumin, and total cholesterol compared to untreated NPC cats (Table 2); because only two cats were evaluated in the 4000 mg/kg group, statistical analyses were not performed. Administration of 1000 mg/kg was sufficient to improve liver function (Table 2), ameliorate hepatic and Kupffer cell swelling (Fig. 2A), and decrease hepatic cholesterol, sphingomyelin, neutral glycolipids, free sphingosine (Fig. 2, B to D), and GM3 ganglioside storage (fig. S2A).

Cats that received HP β CD (1000 mg/kg SC) had a similar onset of neurological dysfunction and mean survival time (21.8 \pm 6.5 weeks; *P* = 0.8) compared to untreated NPC cats. In contrast, two cats that received 4000 mg/kg showed modest amelioration of neurological disease and survived to 31 and 36 weeks of age, which was longer than in any untreated cat. Mean survival time in cats receiving 8000 mg/kg (35.2 \pm 12.4 weeks; *P* = 0.0003) was significantly greater than in untreated NPC cats. However, the first two cats that received this dose were euthanized at 21 and 25 weeks of age because of the acute onset

of severe dyspnea within 24 hours of the weekly HP β CD administration. These cats had diffuse alveolar damage characterized by thickening of the alveolar septa; hypertrophied type II pneumocytes with multifocal hyaline membrane formation, and neutrophils and lymphocytes within alveolar septa; and proteinaceous fluid, fibrin, neutrophils, and foamy macrophages within alveolar spaces (Fig. 5B). In contrast, pulmonary histology in NPC cats receiving 1000 and 4000 mg/kg showed no alveolar damage, although foamy macrophages remained within the alveolar spaces as are present in untreated NPC cats (Fig. 5A). Therefore, in the remaining three cats receiving 8000 mg/kg, HP β CD was discontinued at \sim 20 weeks of age, an age at which mild ataxia and head tremor were present but cats were otherwise clinically normal (video S2). Remarkably, these three cats went on to survive more than twice as long as untreated cats (45 \pm 8.5 weeks; *P* = 6.4 \times 10⁻⁰⁷) without any further therapy, although they eventually developed neurological signs indistinguishable from untreated NPC cats. Notably, after the discontinuation of therapy, these cats survived an additional 21 weeks, which is the mean survival time of untreated NPC cats.

Evaluation of the brains of 24-week-old cats treated with HP β CD (8000 mg/kg) showed a decrease in cerebrocortical filipin staining (Fig. 6) and improved Purkinje cell survival (Fig. 7). The biochemical study of gangliosides indicated a small reduction in the proportion of GM2 ganglioside in a 21-week-old cat treated with 8000 mg/kg (14%) and in another 31-week-old cat receiving 4000 mg/kg (15%). In a 37-week-old cat that had received HP β CD (8000 mg/kg) until 24 weeks of age, the reduction was no longer present (19%) (Fig. 4A); these data were confirmed by immunohistochemical staining (fig. S3). GM3 ganglioside followed the same trend (Fig. 4A). Although gangliosides were no longer reduced, a decrease in cerebrocortical filipin staining was still observed in this 37-week-old cat (Fig. 6). Finally, the lowest sphingosine concentration in the SC category (Fig. 4C) was observed in the 21-week-old treated cat administered HP β CD (8000 mg/kg).

These studies show that SC administration of 1000 mg/kg was sufficient to improve body weight and hepatic disease but had no effect on neurological disease or survival time. In contrast, SC administration of 8000 mg/kg improved weight gain and hepatic disease, onset and severity of neurological dysfunction, cholesterol (and, marginally, ganglioside storage), and survival time. Unfortunately, pulmonary toxicity limited continued long-term peripheral administration of HP β CD at or above this concentration.

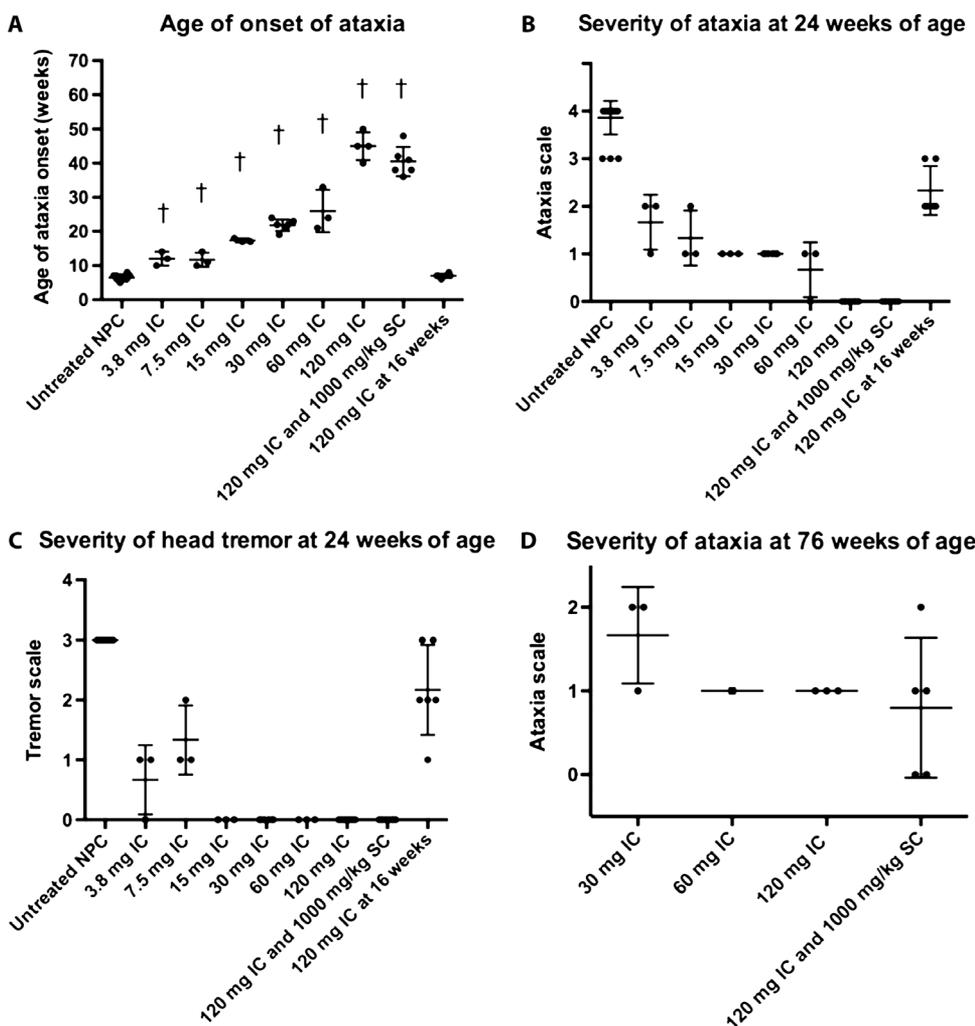


Fig. 1. Age of onset and severity of ataxia in untreated NPC cats and NPC cats administered IC HP β CD. (A) A dose-related effect on the age of onset of ataxia was noted in NPC cats receiving IC HP β CD compared to untreated NPC cats ($^1P < 0.05$). (B to D) At 24 weeks of age, the severity of ataxia (B) and head tremor (C) was diminished in cats dosed presymptomatically; individual cats receiving 60 mg of HP β CD or more showed no ataxia or head tremor at an age when untreated NPC cats were euthanized because of disease progression. No cats receiving 15 mg of HP β CD or greater developed head tremor. At 76 weeks of age, only cats receiving 30 mg of HP β CD or greater were alive, and these cats showed mild to moderate ataxia. Cats first administered HP β CD at 16 weeks of age (postsymptomatically) showed the same onset of ataxia as untreated cats (A), with individual cats showing less ataxia and head tremor at 24 weeks of age compared to untreated NPC cats (B and D). Ataxia was graded on a 0 to 4 scale with 0, none; +1, mild ataxia; +2, moderate ataxia resulting in falling when running; +3, severe ataxia resulting in falling when walking; and +4, no longer able to stand. Head tremor was graded on a 0 to 3 scale with 0, none; +1, mild; +2, moderate; and +3, severe.

Intracisternal HP β CD resulted in neurologically normal cats at 24 weeks of age

To overcome the observed pulmonary toxicity, HP β CD was administered IT at the cisterna magna (IC) to evaluate the efficacy and safety of drug delivered directly to the CNS. A dose of 120 mg, equivalent to an approximate dose of 4000 mg/kg brain weight, was initially evaluated. After administration to seven 24-week-old normal control cats, CSF was sampled at 0.25 ($n = 2$), 1 ($n = 2$), 4 ($n = 3$), and 24 hours ($n = 3$), demonstrating a maximum concentration (C_{max}) = 11645 μ g/ml, half-

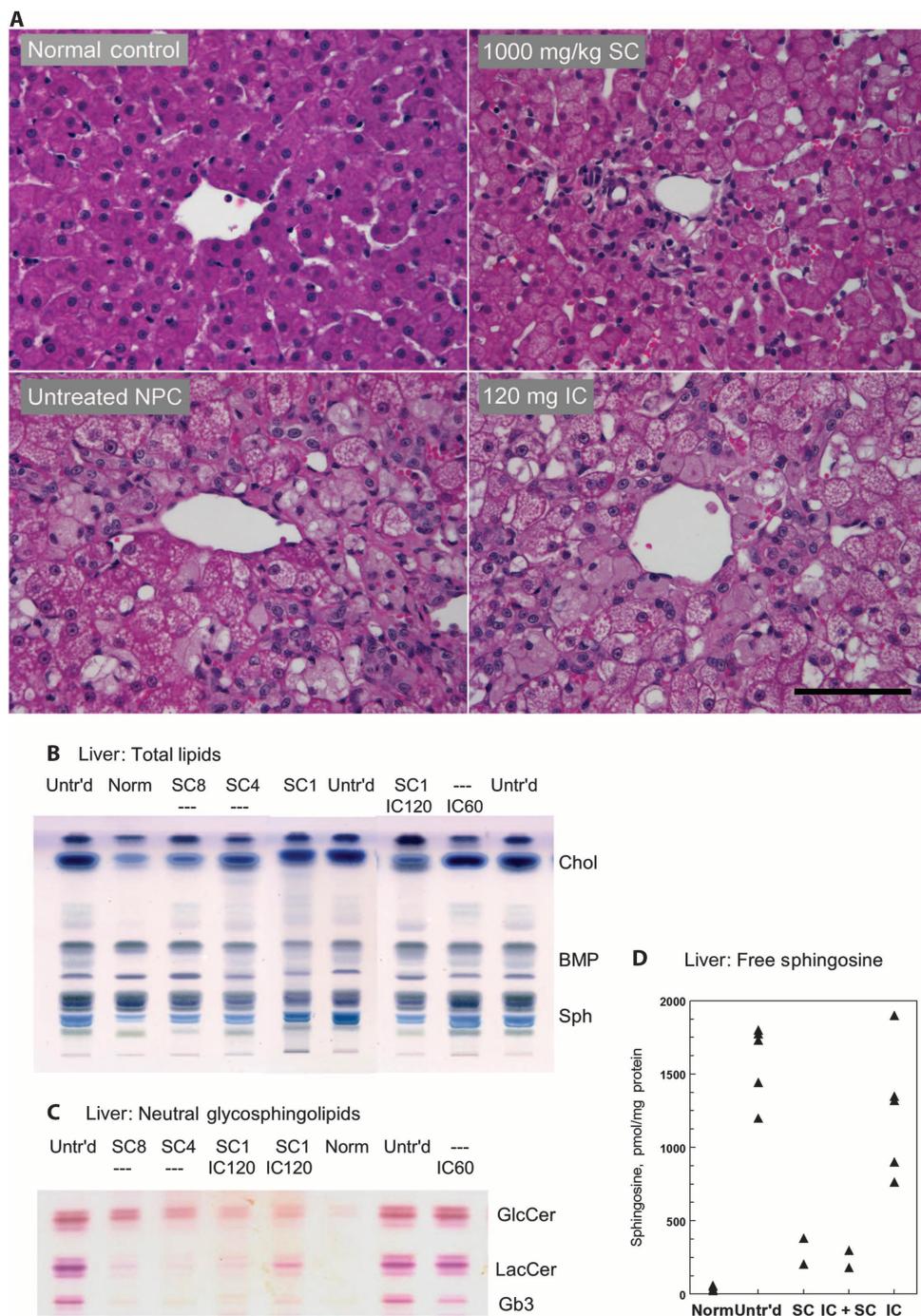
life ($t_{1/2}$) = 0.7 to 2.6 hours, and area under the curve (AUC) = 28614 μ g-hour/ml (Table 3). High concentrations of HP β CD were also observed in cerebellum and cerebrum, up to 689 and 418 μ g/ml, respectively, within 4 hours after IC administration.

NPC cats were treated with 120 mg of IC HP β CD beginning at 3 weeks of age and repeated every 14 days thereafter. Remarkably, these cats were neurologically normal at 24 weeks of age and showed only mild ataxia at 76 weeks of age (videos S3 and S4 and Fig. 1, A to D). In the brain of four cats euthanized at 24 weeks of age (by filipin staining and immunostaining), there was a marked reduction in storage of unesterified cholesterol and GM2 ganglioside (Figs. 3 and 6 and fig. S3), and Purkinje cell loss was completely abrogated (Fig. 7). Purkinje cell numbers were significantly greater than those found in untreated NPC cats ($P = 0.02$). Indeed, upon analysis of hematoxylin and eosin (H&E)-stained sections, treated cats were indistinguishable from normal animals (Fig. 8A). Additionally, granular cell layer thickness was improved, cholesterol and ganglioside storage was decreased, astrogliosis of the cerebellar gray and white matter was reduced, and cerebellar white matter appeared normal (Fig. 3 and fig. S4). Biochemical study of the ganglioside patterns (Fig. 4A) demonstrated drastically reduced storage of gangliosides GM2 (5.3%) and GM3 (4.6%), with unchanged concentrations of the major brain gangliosides. However, there was no improvement in serum albumin, and ALT and cholesterol concentrations were only slightly decreased when compared to untreated NPC cats (Table 2). Liver histology and lipid biochemistry were similar to the findings in untreated NPC cats (Fig. 2).

Additionally, one cohort of NPC cats was treated with HP β CD (a combination of both 120 mg IC and 1000 mg/kg SC) (which contained no allopregnanolone) (Table 1). These cats were neurologically normal at 24 weeks of age and either remained normal or showed only mild ataxia at 76 weeks of age (Fig. 1, A to D). However, they also maintained serum ALT activity and albumin concentrations similar to that found in normal cats, accompanied by significant decreases in serum cholesterol when compared to untreated NPC cats (Table 2; $P = 0.002$). Biochemical analysis of liver showed a marked decrease of cholesterol, sphingomyelin, GM3 ganglioside, neutral glycolipids, and free sphingosine concentrations (Fig. 2, B and C). In the brain, similar results as described for IC treatment alone were observed, with strong decreases in filipin-stained cholesterol, GM2

Fig. 2. Liver histology and biochemistry in NPC cats administered SC or IC HPβCD.

(A) Untreated NPC cats showed severe vacuolization of hepatocyte and Kupffer cell cytoplasm that was ameliorated by HPβCD (1000 mg/kg) but was unaffected by 120 mg of IC HPβCD. All cats were 24 weeks of age. Scale bar, 100 μm. (B) High-performance thin-layer chromatography (HPTLC) of total lipids in 2-mg tissue samples. Migration in chloroform/methanol/H₂O (65:25:4); stained with anisaldehyde spray. The prominent storage of cholesterol and sphingomyelin was greatly reduced in all SC-treated cats but remained unchanged with IC administration. (C) HPTLC showing a similar effect of treatment on neutral glycosphingolipids from 5-mg tissue samples after saponification of total lipids. Migration as in (B); stained with orcinol-H₂SO₄ spray. (D) Free sphingosine concentrations [measured by high-performance liquid chromatography (HPLC)] that were increased ~50-fold in untreated NPC cats decreased about five times after SC HPβCD treatment. For the SC graph, both cats received HPβCD (8000 mg/kg). For the IC graph, data from cats receiving 15, 30, and 60 mg were included because there was no dose effect. Normal values: 37 ± 15 pmol/mg protein (mean ± SD, n = 7). Untr'd, untreated; Norm, normal; SC8, SC4, SC1, subcutaneous, 8000, 4000, or 1000 mg/kg; IC120, IC60, intracasternal, 120 or 60 mg; Chol, unesterified cholesterol; BMP, bis(monoacylglycerol) phosphate; Sph, sphingomyelin; GlcCer, glucosylceramide; LacCer, lactosylceramide; Gb3, globotriaosylceramide.



and GM3 gangliosides (but not in the major gangliosides GM1, GD1a, GD1b, and GT1b), lactosylceramide, and sphingosine, and there was no loss of Purkinje cells (Figs. 4 and 8).

The above studies illustrate the ability of 120 mg of IC HPβCD to ameliorate neurological disease, brain biochemical abnormalities, and Purkinje cell loss when treatment is initiated before the onset of neurological deficits. Because human NPC patients are most often diagnosed after the onset of symptoms, we next determined the effects of instituting therapy when neurological dysfunction was already present.

For this, a cohort of NPC cats was administered 120 mg of HPβCD IC every 14 days beginning at 16 weeks of age, an age at which moderate ataxia (+2) and tremor (+2) exist. All eight NPC cats treated in this manner showed either no progression or slowed progression of clinical signs when evaluated at 24 weeks of age (video S5 and Fig. 1, B and C). Histological and biochemical evaluation of these cats showed an accumulation of cerebrocortical cholesterol, which was similar to that found in untreated NPC cats (Fig. 6). The concentration of GM2 ganglioside (11.7 ± 0.4% of total gangliosides) was clearly less than at

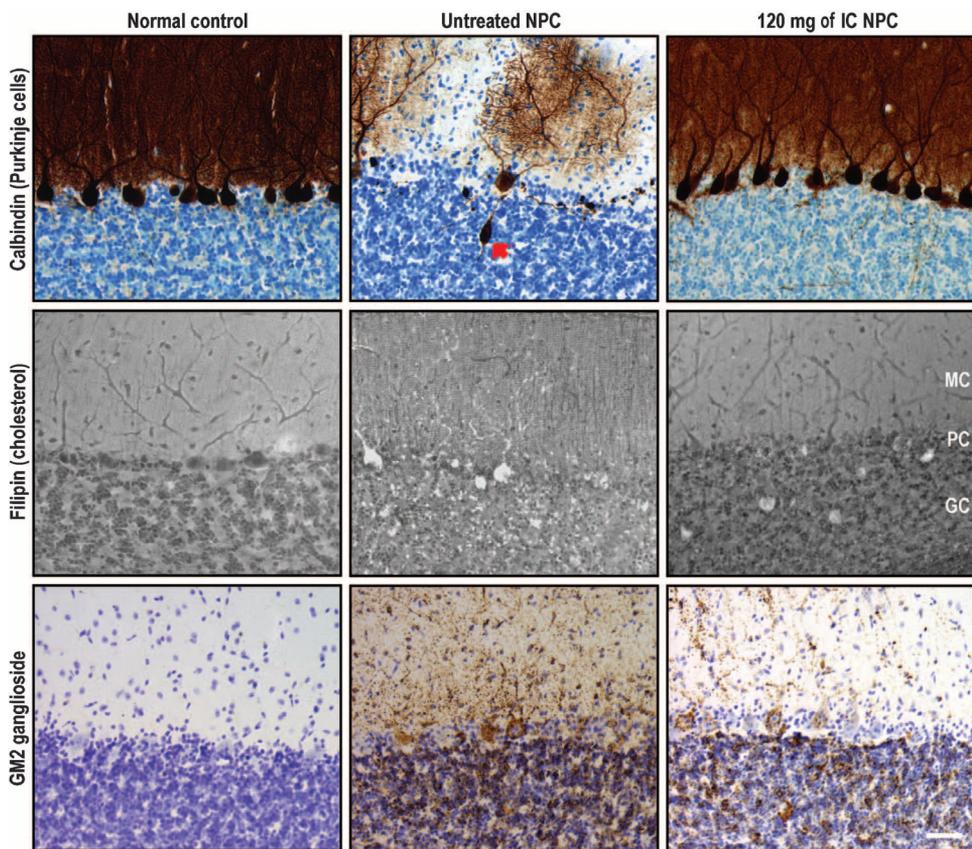


Fig. 3. Calbindin and GM2 immunohistochemical staining and filipin staining of cerebellum from normal control untreated NPC cats and HP β CD-treated NPC cats. (Top row) Immunohistochemical calbindin labeling of Purkinje cells (PCs), whose cell body and dendritic arbor are highlighted in brown. **(Middle row)** Filipin labeling of unesterified cholesterol, accumulation of which is noted by white cytoplasmic staining. **(Bottom row)** GM2 accumulation (dark brown punctae). Normal control column depicts a full complement of PCs lacking cholesterol and GM2 storage, typical of a normal cat at 25 weeks of age. Untreated NPC column shows a large axonal spheroid (red arrow) in one of the remaining PCs within this 25-week-old NPC cat as well as abundant storage of cholesterol and GM2 gangliosides present in all remaining PCs. Treatment of an NPC1 cat with 120 mg of IC HP β CD (third column) resulted in substantial PC rescue as well as decreased cholesterol and GM2 ganglioside accumulation within PCs of this 29-week-old cat. MC, molecular cell layer; GC, granular cell layer. Scale bar, 50 μ m.

the age when treatment began and not much higher than in 4-week-old untreated cats (Fig. 4, A and D). Evaluation of Purkinje cells also suggested that loss of these cells was not as pronounced as that found in untreated cats (Fig. 7); however, quantification studies identified no difference between cats treated postsymptomatically and untreated NPC cats (Fig. 8).

IC HP β CD improved neurological function and survival time in cats with NPC disease

To determine the effect of different doses of IC HP β CD on neurological signs of NPC disease in cats, cohorts were administered doses from 3.8 to 60 mg beginning at 3 weeks of age, and doses were repeated every 14 days thereafter (Table 1). The age of onset of ataxia and the severity of neurological dysfunction at 24 weeks of age varied directly with the administered dose (Fig. 1, A to C). A dose of 3.8 mg of HP β CD IC or greater resulted in a delay in the onset of ataxia and

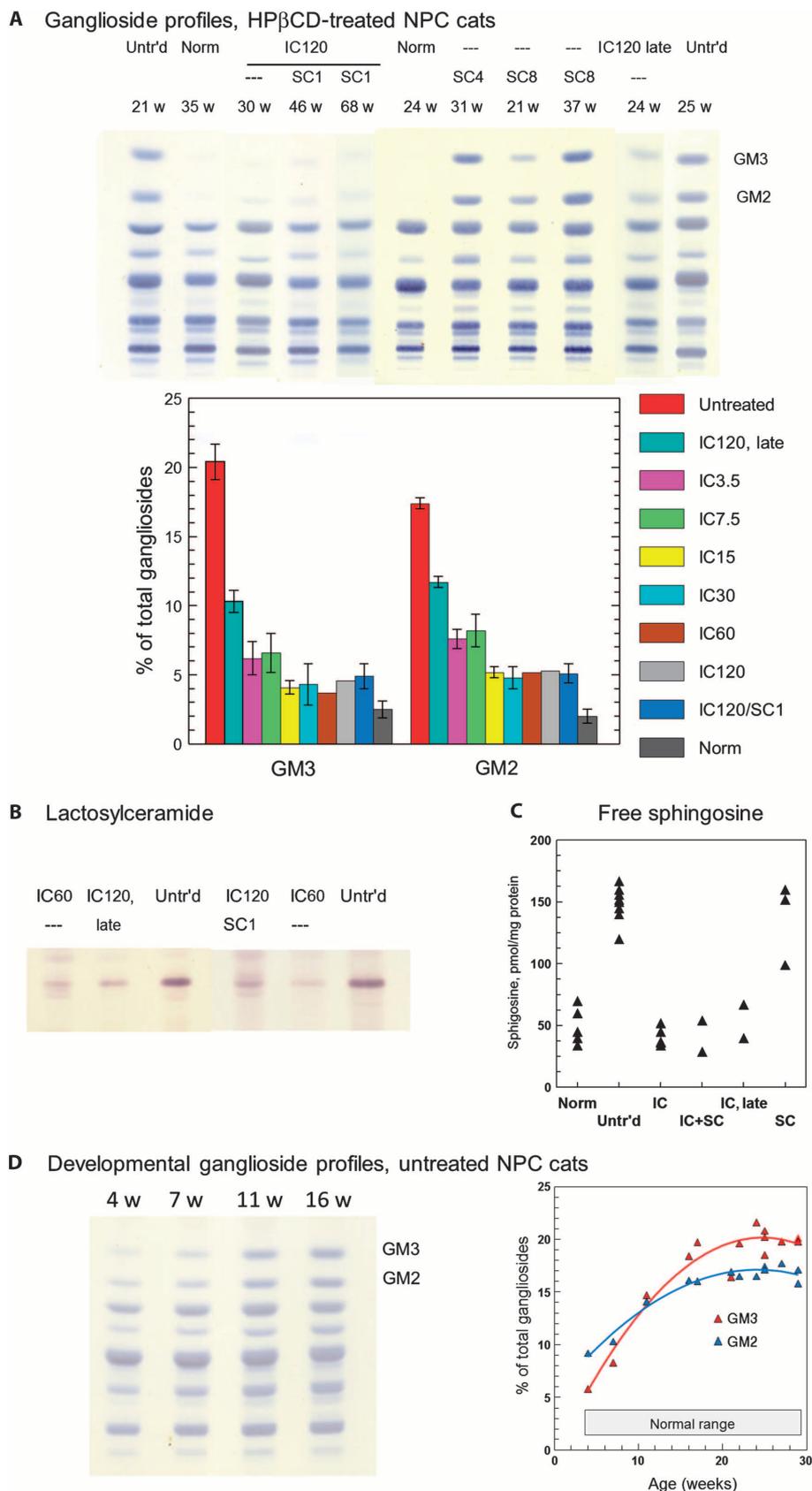
less severe clinical signs at 24 weeks of age when compared to untreated NPC cats. Doses of 15 mg or greater alleviated all head tremor. Doses of 30 mg or greater resulted in survival to at least 1.5 years of age and the development of only mild to moderate ataxia. Doses of 60 mg or greater resolved ataxia in cats at 24 weeks of age (the median age at which untreated cats are euthanized because of the inability to walk or maintain sternal recumbency). No significant differences in outcome measures could be determined between cats dosed with 60 or 120 mg of HP β CD, although, in this study, cats were not evaluated beyond 76 weeks of age.

To evaluate the histological and biochemical effects of dose, two or more cats in each group were sacrificed at 24 weeks of age to evaluate brain pathology and biochemistry (Figs. 4 and 6 to 8). Cerebrocortical neurons showed the greatest amount of filipin staining in cats receiving 3.8 mg and the least staining in cats receiving 30 mg or greater (Fig. 6). This trend held true for ganglioside accumulation as well, with those NPC cats receiving 30 mg or higher exhibiting less storage of GM2 gangliosides than those cats receiving less than 30 mg of IC HP β CD (Fig. S3). Although there was some Purkinje cell loss at lower doses, doses of 30 mg or greater resulted in Purkinje cell preservation (Figs. 7 and 8). Biochemical studies showed that increasing doses of HP β CD were associated with decreases in cerebral GM2 ganglioside concentrations, although this effect plateaued at 15 mg or greater (Fig. 4A).

One or more cats in each IC-treated group were monitored long term and euthanized only when they were nonambulatory and no longer able to remain in sternal recumbency without support, or when they reached 76 weeks of age. Cats receiving 3.8, 7.5, or 15 mg survived to 49, 62, and 66 weeks of age, respectively. In contrast, all cats in treatment groups receiving 30 mg or greater survived the 76-week observation period (Fig. 1D). Neurological dysfunction in cats receiving 30 mg or more progressed slowly, and at 76 weeks of age, these cats showed only mild or moderate ataxia with no evidence of tremor (Fig. 1D). Male cats from the 120 mg IC group are currently over 2 years of age and are breeding and producing kittens.

Four cats that began treatment postsymptomatically (120 mg of IC HP β CD at 16 weeks of age) were observed beyond the 24-week observation period. These cats survived significantly longer than untreated NPC cats (43.5 ± 5.8 weeks of age; $P = 9.06 \times 10^{-11}$). Three cats were euthanized because of inability to maintain sternal recumbency. One cat developed severe diarrhea and was euthanized at 42 weeks of age, although it remained able to walk at this age. The diarrhea was

Fig. 4. Cerebral gray matter lipids in normal control untreated NPC cats and NPC cats administered IC HPβCD. (A) Upper panel: HPTLC of total gangliosides (from 3-mg tissue samples), showing striking and selective reduction of GM3 and GM2 in NPC cats administered IC HPβCD; the ganglioside patterns remained essentially unchanged in NPC cats administered SC HPβCD [migration in chloroform/methanol/0.2% CaCl₂ (55:45:10); stained with resorcinol-HCl spray]. Lower panel: Quantitative data (24- to 26-week-old cats; GM3 and GM2 expressed as percent of total gangliosides). IC treatment at 7.5- and 3.5-mg doses appeared less efficient at reducing GM2 and GM3 than did 15-mg or higher doses of HPβCD. (B) Lactosylceramide concentrations (HPTLC from 10-mg tissue samples) were reduced in cats treated with IC HPβCD. (C) Free sphingosine concentrations (measured by HPLC, expressed as pmol/mg protein) were normalized in all IC-treated cats. (D) HPTLC of total gangliosides (from 3-mg tissue samples) of untreated NPC cats at various ages (left) and developmental profiles of GM2 and GM3 storage (right). Normal proportions (percent of total gangliosides) are 2.0 ± 0.5% for GM2 and 2.5 ± 0.6% for GM3 (mean ± SD, n = 5).



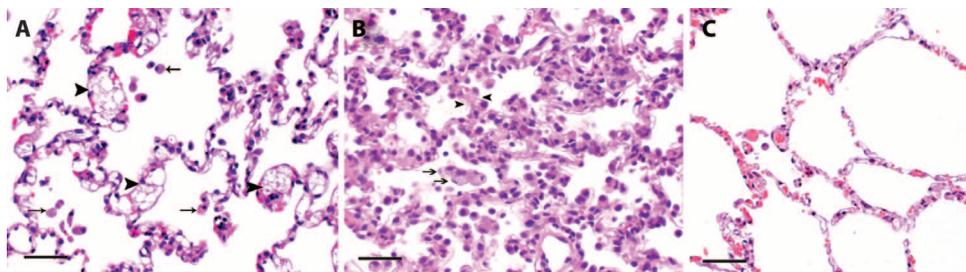


Fig. 5. Pulmonary histology from 6-month-old untreated NPC cats and NPC cats administered HPβCD (8000 mg/kg SC). (A) In untreated NPC cats, the alveolar septa were expanded by foam cells (black arrowheads) as well as by macrophages containing larger irregular clear vacuoles. Alveolar spaces similarly contained foam cells (black arrows). (B) Cats given HPβCD (8000 mg/kg SC) had evidence of acute to subacute diffuse alveolar damage. Alveolar spaces contained abundant proteinaceous fluid with wispy strands of fibrin, foamy macrophages, and neutrophils. The alveolar septa were lined by hypertrophied type II pneumocytes with multifocal hyaline membrane formation. The septa were congested and contained foamy macrophages (black arrows), neutrophils, and lymphocytes. Arrowheads denote thickened alveolar septae. No evidence of alveolar damage was seen in the other treatment groups. (C) Lung from a normal control cat. Scale bar, 100 μm .

associated with an endemic corona virus in the animal colony, which had also required euthanasia of normal control cats.

The data suggested that a 30-mg dose of IC HPβCD or greater was sufficient to profoundly influence disease progression. PK of this dose revealed $C_{\text{max}} = 15,400 \mu\text{g/ml}$, $t_{1/2} = 3.22$ hours, and $\text{AUC} = 23,100 \mu\text{g}\cdot\text{hour/ml}$, which were not significantly different from PK data for the 120-mg dose (Table 3). The PK results suggested that the 30-mg IC dose was sufficient to achieve maximal exposure in the CSF of cats. Brain parenchymal concentrations of HPβCD at this dose were not determined.

SC and IC HPβCD administration caused injection site inflammation and increased the hearing threshold

We next assessed potential safety issues associated with both SC and IC HPβCD administration. A dose of HPβCD (8000 mg/kg SC) resulted in dose-limiting pulmonary toxicity as described above. However, 1000, 4000, and 8000 mg/kg injected SC each produced increasing pain at the injection site with advancing age. Cats receiving both SC and IC HPβCD developed a progressively hunched posture and a short-strided gait in the thoracic limbs that began between 52 and 76 weeks of age. Magnetic resonance imaging (MRI) of the shoulder of these cats revealed evidence of cellulitis, myositis, and arthritis limited to the shoulder joints (fig. S5). SC administration of HPβCD was, therefore, discontinued in these cats after 76 weeks of age. This hunched posture was not seen in cats treated with IC HPβCD alone.

Although hearing thresholds of untreated NPC cats were not significantly different from unaffected cats at 24 weeks of age (Fig. 8B), dose-dependent elevations in mean hearing threshold in cats receiving SC HPβCD were observed [1000 mg/kg = 87 dB SPL (sound pressure level); 4000 mg/kg = 95 dB SPL; and 8000 mg/kg = 112 dB SPL]. In cats receiving IC HPβCD, a significant elevation in hearing threshold was seen in groups of cats receiving either 7.5 or 120 mg IC compared to untreated NPC cats (Fig. 8B). Individual cats receiving 15 mg of HPβCD or greater could be found with hearing threshold greater than or equal to 90 dB SPL, although a dose-dependent elevation in hearing threshold between group means did not reach significance. The elevation in hearing threshold was more pronounced in cats evaluated at 76 weeks of age where groups receiving 30 mg of IC HPβCD or

greater had significant elevation in hearing threshold with many having thresholds equal to or greater than 125 dB, which was the limit of sound generation in the equipment used (Fig. 8C). These results in NPC cats confirm our previous results in normal cats (36), that both SC and IC HPβCD elevate hearing threshold and that continued administration resulted in more profound hearing loss.

DISCUSSION

Cyclodextrin therapy for NPC disease was first evaluated in 2001 when it was found that intraperitoneal HPβCD (500 mg/kg given three times per week) lowered liver unesterified cholesterol and delayed the onset of continuous extensor tremor of the limbs in *Npc1*^{-/-} mice (26). In a subsequent study,

a single dose of SC-administered allopregnanolone (25 mg/kg) dissolved in a 20% HPβCD solution (4000 mg/kg) increased Purkinje cell and granule cell survival, reduced brain ganglioside accumulation, and nearly doubled the life span of *Npc1*^{-/-} mice (43). Although the substantial positive effects were attributed to the reconstitution of allopregnanolone in the *Npc1*^{-/-} mouse brain (43), follow-up studies by two laboratories independently found that HPβCD (4000 mg/kg SC) alone was sufficient to reduce storage of cholesterol and gangliosides and to positively affect NPC-related neurological disease (30, 31, 44). Our data in the feline model support evidence of a dose-related effect of SC HPβCD on NPC disease. HPβCD (1000 mg/kg SC), in the presence or absence of allopregnanolone, had substantial positive effects on hepatic disease in feline NPC disease but had no effect on neurological disease. In contrast to mice, HPβCD at a dose of 4000 mg/kg SC resulted in only modest disease amelioration, whereas HPβCD at a dose of 8000 mg/kg SC resulted in reduced NPC-associated neurological disease and increased survival time. However, 8000 mg/kg also resulted in life-threatening pulmonary toxicity that was not anticipated, because previous studies had not involved chronic administration of HPβCD at this dose (45–48). Histological evaluation confirmed the proliferation of cuboidal epithelial cells (type II pneumocytes) consistent with lung damage (49). It is possible that the pulmonary toxicity, and injection site inflammation, associated with HPβCD may have been due to differences in the degree of substitution or impurities present in the HPβCD formulation and not directly due to the cyclodextrin. The powdered form of HPβCD (HPβCD-H107; Sigma-Aldrich) was used for SC injections because high doses were necessary and this was the least expensive formulation available. In contrast, the cell culture system-tested form (HPβCD-C0926; Sigma-Aldrich) was used for all IC injections. Our study cannot rule out whether the degree of substitution or impurities in different drug formulations was responsible for the pulmonary toxicity seen in cats.

Pulmonary disease is a rarely described finding in NPC patients, with greater severity found in NPC2 rather than in NPC1 disease (2, 50–54). In untreated NPC cats, lung histology showed thickened septae and foamy macrophages within the alveolar spaces and septa, changes identical to those seen in the *Npc1*^{-/-} mouse (55). Data from

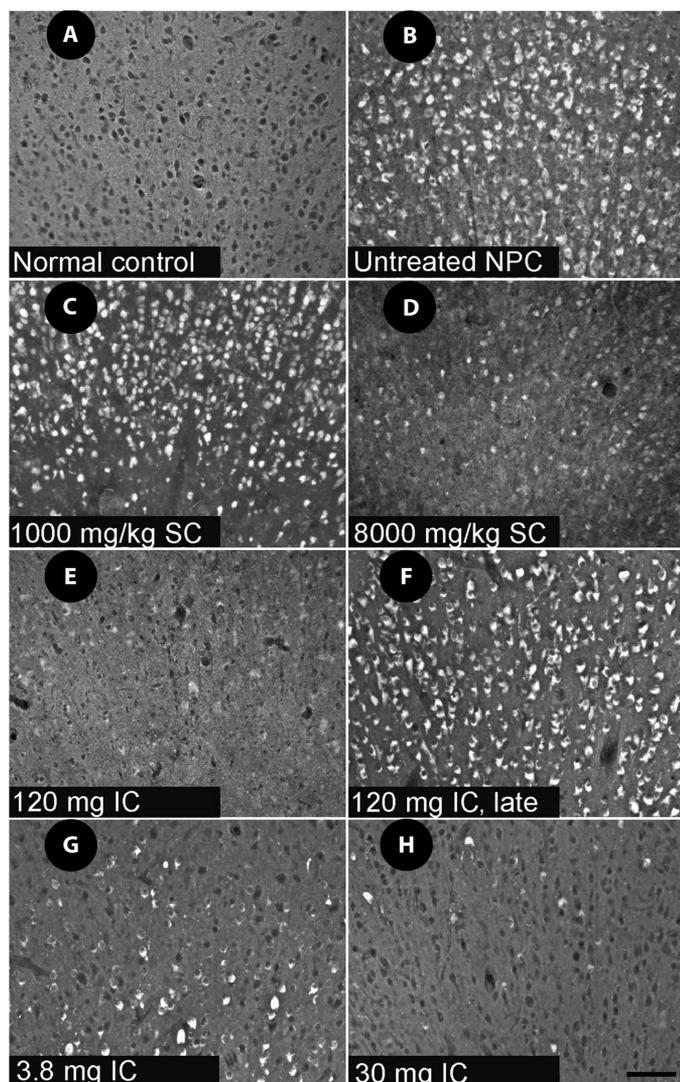


Fig. 6. Filipin staining of marginal gyrus of cerebral cortex in NPC cats. (A and B) Untreated 24-week-old NPC cats (B) showed storage of cholesterol (visualized as white cytoplasmic labeling within cells) in the marginal gyrus of the cerebral cortex, and no cholesterol storage was observed in healthy control animals (A). (C) Cats receiving HPβCD (1000 mg/kg SC) showed no reduction in cholesterol staining. (D) Cats receiving HPβCD (8000 mg/kg SC) showed substantially less cholesterol staining than did untreated NPC cats. Indeed, the example shown is from a 37-week-old cat that had received SC HPβCD until 24 weeks of age, at which time it was discontinued. (E and H) Cats (24 weeks old) receiving 30 mg of IC HPβCD or greater presymptotically showed the greatest amelioration of the cholesterol storage defect. In contrast, treatment of NPC cats with 3.8 mg of IC HPβCD (G) or 120 mg of IC HPβCD administered postsymptotically (F) revealed little effect on stored cholesterol at 24 weeks of age, whereas both 30 and 120 mg of HPβCD administered presymptotically resulted in substantial reductions in cholesterol storage. Scale bar, 100 μ m.

NPC mouse lung showed that HPβCD (4000 mg/kg) did not reverse pulmonary disease (30, 56–58), nor did it decrease unesterified cholesterol concentrations or cholesterol synthesis, suggesting that macrophages in the lung did not have access to plasma HPβCD (56). It is

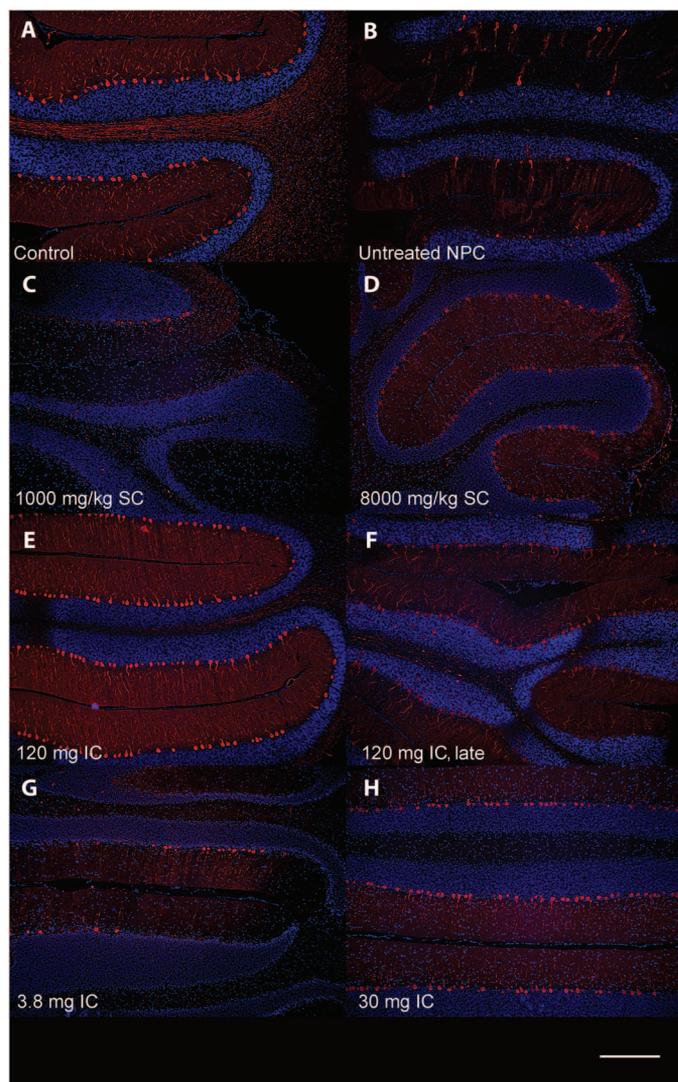


Fig. 7. Calbindin immunofluorescence of cerebellar paravermis in 6-month-old NPC cats. (A and B) Untreated NPC cats (B) showed a severe loss of Purkinje cells compared to normal control cats (A). (C and D) SC HPβCD administration at a dose of 8000 mg/kg resulted in increased Purkinje cell survival (D), whereas cats receiving HPβCD (1000 mg/kg SC) were indistinguishable from untreated NPC cats (C). (H) Cats treated with IC HPβCD at 30 mg or greater resulted in substantial Purkinje cell survival. In contrast, 3.8 mg of IC HPβCD (G) or 120 mg of IC HPβCD given either presymptotically (E) or postsymptotically (120 mg IC, late) (F) resulted in small but nonsignificant increases in Purkinje cell number compared to untreated NPC cats. Scale bar, 200 μ m.

interesting to note that treated cats have thus far shown no evidence of developing pneumonitis as seen in *Npc1*^{-/-} mice; however, foamy macrophages continue to be evident in lungs from cats treated with SC HPβCD.

Although it is clear that administration of SC HPβCD positively affects CNS disease in both NPC mice and cats, it apparently does so without blood-brain barrier penetration (26, 32). Our current study shows that SC doses of 4000 and 8000 mg/kg resulted in very low CSF concentrations (~20 μ g/ml; ~14.3 μ M) measured 60 min after administration

Table 3. PK of HPβCD after IC or IV administration in normal control cats. NC, not calculated due to below detectable concentrations for some samples.

Analyte	Route of administration	Dose	Matrix	C ₀ (μg/ml)	AUC _{0-∞} (μg-hour/ml)	AUC _{0-24h} (μg-hour/ml)	t _{1/2} (hour)	CL/F (ml/hour-kg)	Vd _{ss} (ml/kg)
HPβCD (Sigma)	IC	120 mg	CSF	11,645	28,614	—	0.7–2.6	—	—
HPβCD (Kleptose)	IC	30 mg	CSF	15,400	23,300	23,100	3.22	1.29	4.14
HPβCD (Kleptose)	IV	1000 mg/kg	CSF	13.1	NC	346	NC	NC	NC
HPβCD (Kleptose)	IV	1000 mg/kg	Plasma	3,760	4,660	6,770	1.11	215	336

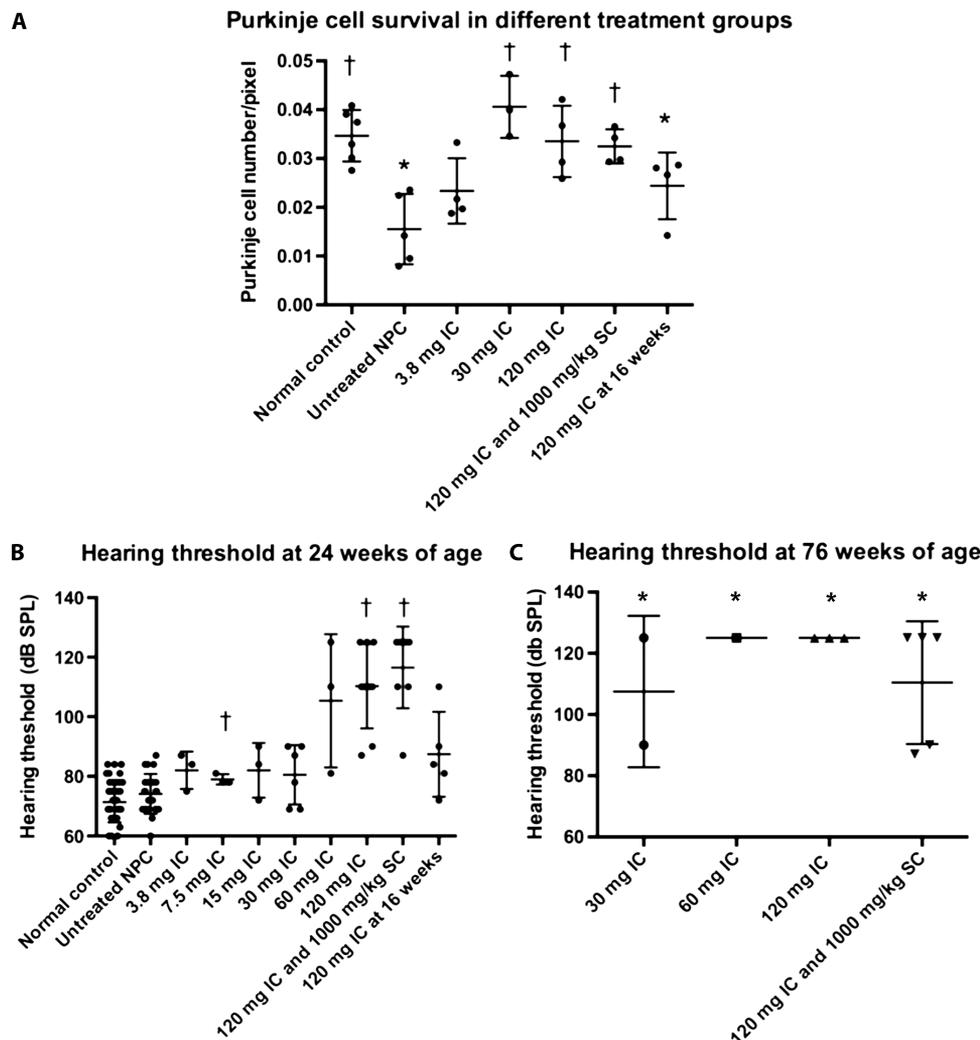


Fig. 8. Purkinje cell quantification and hearing threshold in NPC cats administered IC HPβCD. (A) Untreated 24-week-old NPC cats showed significantly fewer Purkinje cells per unit length compared to age-matched normal control cats (* $P < 0.05$). Cats (24 weeks old) receiving 30 mg of IC HPβCD or greater showed significantly greater Purkinje cell numbers compared to untreated NPC cats ($^{\dagger}P < 0.05$). In contrast, 24-week-old cats treated postsymptomatically with 120 mg of IC HPβCD at 16 weeks of age showed significantly fewer Purkinje cells compared to normal control cats (* $P < 0.05$). (B) Untreated 24-week-old NPC cats showed no significant difference in hearing threshold compared to normal control cats. In contrast, cats receiving 7.5 or 120 mg of HPβCD developed significant increases in hearing threshold compared to untreated NPC cats ($^{\dagger}P < 0.05$). Individual cats receiving lower doses of HPβCD also showed elevated hearing thresholds, although, as a group, these were not significant. (C) At 76 weeks of age, all NPC cats receiving 30 mg of HPβCD or greater showed significant elevations in hearing threshold. * $P < 0.001$.

and a brain-to-plasma ratio of 0.7%, which is similar to what was found in mice (40), and approximates the serum/CSF ratio reported for human albumin (59, 60). To more fully evaluate the PK of this drug, we administered control cats a single dose of HPβCD (1000 mg/kg) intravenously (IV) and determined CSF and plasma concentrations (Table 3). This IV dose resulted in a maximal serum concentration similar to what was found 60 min after SC administration of HPβCD (8000 mg/kg). The CSF concentration of 13.1 μg/ml was also similar, again indicating poor CNS penetration of HPβCD and a serum/CSF ratio of 0.3%. Therefore, one proposed explanation for the clinical efficacy seen in NPC cats receiving high SC doses is that, similar to serum albumin (40), HPβCD crosses the blood-brain barrier in small quantities through fluid-phase transcytosis and thus requires high serum concentrations of HPβCD to mitigate CNS disease. The evaluation of brain parenchymal concentrations of HPβCD after SC administration may further our understanding of whether serum HPβCD concentrations affect CNS disease directly or via indirect effects on brain endothelial cells as has previously been proposed (32, 61, 62).

Our results demonstrate that direct administration of HPβCD into the CNS avoids the need for high serum concentrations and associated pulmonary toxicity. A dose-related effect on the age of onset of ataxia, severity of ataxia, brain cholesterol and ganglioside storage, and Purkinje cell survival was identified in cats treated before the onset of symptoms. Improvement in neurological function and Purkinje cell survival were always accompanied by concomitant decreases in cholesterol and ganglioside storage. Doses of 30 mg or greater resulted in the most profound amelioration of biochemical

disease, and although significant differences in Purkinje cell numbers in cats administered 30, 60, or 120 mg of IC HP β CD could not be identified, age of onset of ataxia and severity of ataxia suggest that 120 mg was the most efficacious. On the basis of these data, the maximally effective dose of IC HP β CD has not been determined. Planned studies include the administration of doses above 120 mg, the evaluation of neurological function in cats beyond 76 weeks of age, the evaluation of cerebellar pathology in cats at 76 weeks of age and beyond, and the effect of IC HP β CD administration on CNS tissue outside of the cerebellum.

Postsymptomatic therapy prolonged life span and slowed the progression of neurological dysfunction. However, no cat in this group survived beyond 1 year of age, and no increase in Purkinje cell numbers was found when compared to 24-week-old untreated NPC cats. NPC cats euthanized at 16 weeks of age, when cats were first administered HP β CD, were not available for histological comparison. Postsymptomatically treated cats showed biochemical evidence of decreased ganglioside storage compared to age-matched untreated NPC cats, but the decrease was less profound than in cats treated presymptomatically. Indeed, immunohistochemistry showed less evidence of decreased gangliosides in the brains of postsymptomatically treated cats. Similarly, little improvement in concentrations of cholesterol storage in the late endosomal/lysosomal compartment was identified by histochemical staining with filipin. These data suggest that presymptomatic therapy is most effective at slowing disease progression and that cholesterol and ganglioside storage may be more difficult to correct postsymptomatically. Biomarkers that are currently being validated to correlate with clinical improvement may shed light on whether decreases in cholesterol, sphingolipids, or some other metabolite best correlate with Purkinje cell survival and clinical improvement.

An elevated hearing threshold was identified as an adverse effect in cats administered either SC or IC HP β CD. Hearing loss is not a characteristic of feline NPC disease (34, 36); however, HP β CD administration resulted in a dose- and duration of treatment–dependent increase in hearing threshold in both affected and control cats (36). Studies performed in mice confirmed that HP β CD-induced elevations in hearing threshold were accompanied by loss of outer hair cells within the cochlea, presumably due to changes in membrane composition and integrity and consequent effects on the outer hair cell motor protein prestin (63). Our studies in the cat indicate that chronic HP β CD at therapeutic doses is always accompanied by severe hearing loss. These findings have important implications for clinical use in human patients where deafness may be an expected outcome of therapy. Potential methods to mitigate HP β CD-mediated hearing loss including adjunct therapy, limiting access of HP β CD to the inner ear, using other cyclodextrins that may be less toxic, or managing hearing loss with either hearing aids or cochlear implants are being evaluated.

A complete understanding of how HP β CD reduces neuronal storage of cholesterol and sphingolipids and results in Purkinje cell survival remains to be determined. In vitro studies of HP β CD in cultured murine *Npc1*^{-/-} neurons show a concentration-dependent effect on cholesterol storage (15, 64) with low doses (0.1 mM) releasing cholesterol from the late endosomal/lysosomal compartment to the endoplasmic reticulum; higher doses (5 to 10 mM) are toxic and deplete cholesterol from the plasma membrane. Intraventricular administration of HP β CD in the *Npc1*^{-/-} mouse resulted in Purkinje cell survival and was accompanied by evidence of intracellular cholesterol mobilization from the lysosome to the metabolic pool of the cytosolic compartment [suppressed cholesterol synthesis, elevated cholesterol esters, suppressed SREBP2 target genes, and

activated LXR control genes] (65, 66). In the cat and mouse models, as well as in human patients, HP β CD administered directly into the CSF transiently increased both plasma and CSF 24(S)-hydroxycholesterol [24(S)-HC] concentrations (40). Given that conversion of unesterified cholesterol to 24(S)-HC is the principal route of excretion of cholesterol from the CNS, its elevation indicates that cholesterol flux through the lysosome to the cytosolic compartment was normalized after treatment with HP β CD. However, other studies also suggest that cyclodextrins may directly interact with sphingolipids or other membrane constituents as well as cholesterol (67). Genetic reductions of complex gangliosides in *Npc1*^{-/-} mice have been shown to be accompanied by reduced intraneuronal cholesterol sequestration (68, 69). Finally, cyclodextrins may function through substrate-independent mechanisms such as modulation of autophagy (70) or stimulation of exocytosis (71). Indeed, dose-related clinical effects of HP β CD were accompanied by parallel changes in cholesterol, sphingolipids, and Purkinje cell survival in the feline model, and we are currently evaluating effects on autophagy.

Direct injection of CSF with HP β CD ameliorates neuronal storage of cholesterol and gangliosides and improves Purkinje cell survival in both NPC mice (40, 65, 66) and cats, supporting its therapeutic potential in human patients. Here, we used direct injection into the cerebellomedullary cistern of our feline model, but IT injections in humans are typically carried out by lumbar puncture or intraventricular injection. In the current phase 1 trial, HP β CD [Kleptose HPB; degree of substitution (DS), 0.4; average molecular weight (MW), 1400] is being administered by monthly lumbar puncture. Therefore, to increase the translational potential of these studies, we are currently evaluating the safety and efficacy of lumbar injections in the feline model. Also, repeated cisternal administration of HP β CD in cats required general anesthesia using propofol every 2 weeks. Untreated NPC cats were also anesthetized every 14 days to acquire CSF for biomarker evaluation (40), and there was no evidence that disease progression was altered by anesthesia alone. In the current phase 1 trial, only local anesthetic or sedation is being used. Finally, human patients present with learning disabilities, dementia, vertical supranuclear gaze palsy, seizures, dysarthria, and dysphagia in addition to cerebellar ataxia. Unfortunately, dementia and dysarthria are difficult to model in cats, and monitoring seizures by electroencephalography is challenging in awake cats. Because supranuclear gaze palsy and dysphagia were not observed in our feline model, we plan to verify regional cerebrocortical and brain stem effects of HP β CD using histological and biochemical methods. Thus far, we predict that IT therapy with HP β CD will improve cerebellar ataxia in patients and, therefore, could be used to enhance the overall clinical well-being of patients with NPC disease.

MATERIALS AND METHODS

Study design

This was a prospective study. Each treatment cohort (Table 1) was composed of at least three cats to make statistical comparisons, except for the group of cats that received HP β CD (4000 mg/kg SC) (see below). Clinical endpoints for the study were defined before study onset and included (i) inability to walk or maintain sternal recumbency and (ii) uncontrolled diarrhea resulting in dehydration. Cats were euthanized when these endpoints were reached. No outliers were defined, and no cats enrolled were excluded from the study. The objective of the study was to determine

whether direct administration of HP β CD to the IT space at the cerebellomedullary cistern provided greater NPC disease amelioration than peripheral SC administration. First, cohorts of NPC cats were compared that received HP β CD (1000, 4000, and 8000 mg/kg SC) every 7 days starting at 3 weeks of age. Cats were assigned to each cohort in a dose-escalation fashion, with the first treated cohort receiving the lowest dose. Survival data in the two cats in the 4000 mg/kg cohort indicated insufficient effects at this dose, and, therefore, because of the limited number of cats that could be produced, no additional cats were evaluated in this cohort. Pulmonary toxicity limited the continued dosing of cats in the 8000 mg/kg group. Several cohorts of affected NPC cats were evaluated using multiple IT-administered doses. A dose of 120 mg IT, equivalent to an approximate dose of 4000 mg/kg brain weight, was initially evaluated. After data collection from this group, five different groups of cats received 3.8, 7.5, 15, 30, or 60 mg of HP β CD IT, respectively, every 14 days beginning at 3 weeks of age. Cats were assigned to each group by placing pieces of paper with the dose written on them into a container. When cats were born, the investigator drew a piece of paper from the container and assigned cats to each group on the basis of the results. Finally, one group of NPC cats received a combination of HP β CD (1000 mg/kg SC) every 7 days along with 120 mg of HP β CD IT every 14 days starting at 3 weeks of age, and one group of NPC cats received 120 mg of HP β CD IT every 14 days starting at 16 weeks of age. Age-matched cohorts of untreated NPC cats were available for comparison to treated cats up to the typical time of end-stage disease; treated versus untreated cohorts were determined by a coin flip. Efficacy of therapy was evaluated by survival data, neurological examination performed weekly in all cats, body weight determined weekly in all cats, serum collected at various intervals for serum chemistry evaluation to determine the effect of therapy on liver enzymes, and microscopic examination of selected tissues. HP β CD-treated cats were sacrificed at about 24 weeks of age to compare histological and biochemical data to data from age-matched untreated cats. Additionally, a number of treated cats (indicated in Results) were followed long term to assess the effect of HP β CD over time in cats.

Both male and female cats were evaluated in this study. Investigators were not blinded during the administration of HP β CD doses to cats, during the clinical evaluation of cats, or during the evaluation of tissue collected from cats except where specified.

Animals

Cats were raised in the National Referral Center for Animal Models of Human Genetic Disease of the School of Veterinary Medicine of the University of Pennsylvania (NIH OD P40-10939) under National Institutes of Health and U.S. Department of Agriculture guidelines for the care and use of animals in research. The experimental protocol was approved by the University's Institutional Animal Care and Use Committee. Peripheral blood leukocytes from all the cats were tested at 1 day of age for the NPC1 missense mutation using a polymerase chain reaction-based DNA test to identify affected as well as normal, control cats as previously described (20, 33, 34). Body weight was measured, and physical and neurologic examinations were performed weekly from birth until death for all cats. The onset and progression, as well as the severity of signs of neurologic dysfunction were identified. Cerebellar ataxia was graded on a 0 to 4 scale (0, none; +1, mild ataxia; +2, moderate ataxia resulting in falling when running; +3, severe ataxia resulting in falling when walking; and +4, no longer able to stand). Head tremor was graded on a 0 to 3 scale (0, none; +1, mild; +2, moderate; and

+3, severe). Brain stem auditory-evoked response testing was performed using previously described methods (36).

HP β CD formulations and treatment groups

The powdered form of HP β CD (HP β CD-H107; Sigma-Aldrich) was used in all SC administrations, and the cell culture-tested form (HP β CD-C0926; Sigma-Aldrich) was used for all IC administrations except where indicated. Kleptose HPB (DS, 0.4; average MW, 1400) was also used in IC and IV PK experiments and was provided by Janssen Research & Development. All HP β CD was administered in a 20% (w/v) solution dissolved in 0.9% saline (Hospira Inc.) except when administered as a 3% solution dissolved in saline because of the small volume of administration (3.8, 7.5, and 15 mg). In cats receiving 1000 mg/kg SC alone, the 20% solution of HP β CD also contained allopregnanolone (25 mg/kg) (provided by S. Mellon); when HP β CD (1000 mg/kg SC) was administered in combination with IC therapy, allopregnanolone was not included in either dose. The inclusion of allopregnanolone in the cats receiving HP β CD (1000 mg/kg SC) alone was due to these cats being treated soon after the publication of the Griffin *et al.* study (43). Subsequent murine studies, however, have shown that cyclodextrin alone was sufficient to positively affect NPC-related neurological disease (30, 31, 44), and therefore, allopregnanolone was not administered to any other groups of cats. Dosing volumes for SC administration varied from 5 to 40 ml/kg. Dosing volumes for IC injections varied from 0.1 to 1 ml with 20% HP β CD formulations.

Thirteen cohorts of cats were evaluated (Table 1) including normal control cats and untreated NPC cats. Three groups received HP β CD SC every 7 days beginning at 3 weeks of age by tenting a region of skin over the neck and injecting the solution into the SC space. Six groups received HP β CD IT at the cerebellomedullary cistern (IC) every 14 days beginning at 3 weeks of age. One group received a combination of HP β CD (1000 mg/kg SC) every 7 days and 120 mg of HP β CD IC every 14 days beginning at 3 weeks of age. Finally, one group received 120 mg of HP β CD IC every 14 days beginning at 16 weeks of age. All IC dosing and CSF collected were performed in cats anesthetized with propofol (up to 6 mg/kg IV; Abbott Laboratories).

Blood and CSF collection

About 3 ml of blood was collected from either the cephalic or jugular vein at 8, 16, and 24 weeks of age to perform a complete blood count and serum chemistry analysis. About 1 ml of CSF was collected from the cerebellomedullary cistern at the time of each dosing and every 2 weeks from untreated NPC cats. Remaining serum and CSF were frozen at -80°C for biomarker studies.

Histological analysis

For pathological evaluations, cats were sacrificed at ~ 24 weeks of age, an age at which untreated NPC cats can no longer remain sternal and are euthanized. A number of treated affected cats were also observed for a longer period of time to assess the efficacy of HP β CD; these cats were euthanized when they were no longer able to remain sternal or when they reached 76 weeks of age. Euthanasia was performed using an overdose of IV barbiturate. Immediately before euthanasia, cats were given an IV dose of 200 U of heparin to prevent clotting during the tissue harvest. After sacrifice, animals were perfused through the left ventricle with 750 ml of 0.9% cold saline, and samples of brain, liver, and lung were acquired and flash-frozen. After perfusion, tissue samples were collected, sectioned, and dropped-fixed in 4% paraformaldehyde for 48 hours. Transverse

sections of brain at the level of the caudate nucleus and cerebellar nuclei tissue were placed in cold 0.1 M phosphate buffer and shipped overnight to the Walkley laboratory for processing. These sections were dissected into selected blocks, cut on a vibratome (35 μ m), and processed to visualize cholesterol storage (via filipin labeling) and ganglioside storage (via immunohistochemistry or immunofluorescence) using methods previously described (19, 72). The remainder of the brain as well as tissue from other organs were paraffin-embedded, sectioned at 5 μ m, and stained with H&E. For immunohistochemistry, sections on charged slides were deparaffinized through xylenes and graded ethanols, and antigen retrieval was performed in a microwave using Antigen Retrieval Citra Solution (BioGenex). Purkinje cells were identified using rabbit anti-calbindin (Swant) diluted 1:3000, and granule cells (used as a reference) were identified using mouse anti-NeuN (Millipore, #MAB377) diluted 1:500 in Antibody Diluent Reagent Solution (Invitrogen). After a 12-hour incubation at 4°C, unbound primary antibodies were washed off and the sections were incubated for 30 min at 37°C with Alexa Fluor 568- and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen). Two fields from each specimen were imaged at 2.5 \times on a Leitz DMRB fluorescence microscope equipped with a QImaging Retiga-2000DC charge-coupled device camera controlled by iVision-Mac image acquisition and analysis software (BioVision Technologies). Images were analyzed using iVision-Mac. Calbindin-positive Purkinje cells were counted for each specimen, and a line was then drawn along the Purkinje cell layer to determine a total length in pixels. The total number of labeled cells per specimen divided by the total length generated a labeling index for the specimen. The myelin basic protein (MBP) antibody used was a mouse anti-MBP from Abcam (#ab24567). The LAMP1 antibody used was a rabbit polyclonal from Abcam (#ab24170).

Biochemical analysis of lipids

Lipid studies on dissected cerebral cortex, cerebellum, and liver were conducted on frozen tissues, following exactly the same procedures as described in a recent study on NPC cats treated with miglustat (20). Part of the control biological material (untreated NPC cats and normal cats) could thus be shared in both studies. The methodology has been described earlier in more detail (20, 30, 73, 74). Isolation of gangliosides from total lipid extracts was performed by reversed-phase chromatography on Bond Elut C18 100-mg columns. Silica gel high-performance thin-layer plates were from Merck; densitometry of the plates at 580 nm was performed using a Camag TLC II scanner with CATS software. Here, individual ganglioside concentrations were not expressed in nmol/g wet weight as in previous publications, but as percentage of total gangliosides. This mode of expression was elected because it was independent of potential dehydration of the tissue samples during storage and thus reduced methodological variation when comparing the effect of different doses of HP β CD.

Bioanalytical and PK analyses of HP β CD

Plasma and CSF HP β CD were quantified using a reversed-phase ultra-performance liquid chromatography tandem mass spectrometry (MS/MS) method (75). PK of the plasma and CSF concentrations of HP β CD was performed to determine the concentration at zero time ($C_0 = C_{\max}$) by the extrapolation of drug concentration back to the time of dosing the area under the plasma concentration versus time curve ($AUC_{0-\infty}$ and AUC_{0-24h}), terminal half-life ($t_{1/2}$), plasma clearance (CL), and the apparent volume of distribution at steady state (Vd_{ss}) for IV (CSF only) and IT dose groups using the WinNonlin version 5.3 (Pharsight) program.

Statistics

Mean values and SDs were calculated to describe the findings. The unpaired two-tailed *t* test was used to compare data from treated cats to both wild-type and normal cats. Values were considered statistically significant at the $P < 0.05$ level. *P* values are provided where significant differences exist.

SUPPLEMENTARY MATERIALS

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Fig. S1. Weekly mean body weights of normal control, untreated NPC, and NPC cats administered SC or IC HP β CD.

Fig. S2. Liver gangliosides and brain glucosylceramide.

Fig. S3. Immunofluorescence staining of GM2 ganglioside in marginal gyrus of cerebral cortex.

Fig. S4. Immunofluorescence staining of MBP in cerebellar white matter.

Fig. S5. Abnormal posture and MRI of shoulders of 76-week-old NPC cat that received both SC and IC HP β CD.

Video S1. Untreated 21-week-old NPC cat showing tremor (+3), ataxia (+4), and inability to stand without support.

Video S2. NPC cat (22 weeks old) administered HP β CD (8000 mg/kg SC) weekly since 3 weeks of age showing mild head tremor (+1) and ataxia (+1).

Video S3. NPC cat (24 weeks old) administered 120 mg of HP β CD IC biweekly since 3 weeks of age appearing neurologically normal.

Video S4. NPC cat (76 weeks old) administered 120 mg of HP β CD IC biweekly since 3 weeks of age showing no head tremor and mild ataxia (+1).

Video S5. NPC cat (24 weeks old) administered 120 mg of HP β CD IC biweekly since 16 weeks of age showing head tremor (+2) and ataxia (+2).

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Editor's Summary

Cyclodextrin to the rescue

Niemann-Pick type C1 (NPC) disease is a severe hereditary nervous system disorder associated with the storage of cholesterol and other lipids inside nervous tissue. In new work, Vite *et al.* show that injection of the pharmaceutical excipient cyclodextrin into the spinal fluid of cats with naturally occurring NPC disease prevented lipids from accumulating and prevented nervous system disease from developing. The only side effect found was a loss of hearing acuity associated with therapy. This study in the cat model provides critical data on efficacy and safety of cyclodextrin administration directly into the spinal fluid that will be important for advancing this drug into clinical trials.

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