Efficacy and ototoxicity of different cyclodextrins in Niemann–Pick C disease

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Abstract
Objective: Niemann–Pick type C (NPC) disease is a fatal, neurodegenerative, lysosomal storage disorder characterized by intracellular accumulation of unesterified cholesterol (UC) and other lipids. While its mechanism of action remains unresolved, administration of 2-hydroxypropyl-β-cyclodextrin (HPβCD) has provided the greatest disease amelioration in animal models but is ototoxic. We evaluated other cyclodextrins (CDs) for treatment outcome and chemical interaction with disease-relevant substrates that could pertain to mechanism.

Methods: NPC disease mice treated for 2 weeks with nine different CDs were evaluated for UC, and GM2 and GM3 ganglioside accumulation using immunohisto/cytochemical and biochemical assays. Auditory brainstem responses were determined in wild-type mice administered CDs. CD complexation with UC, gangliosides, and other lipids was quantified.

Results: Four HPβCDs varying in degrees of substitution, including one currently in clinical trial, showed equivalent storage reduction, while other CDs showed significant differences in relative ototoxicity and efficacy, with reductions similar for the brain and liver. Importantly, HPβCD and two sulfobutylether-CDs showed efficacy with reduced ototoxicity. Complexation studies showed: incomplete correlation between CD efficacy and UC solubilization; an inverse correlation for ganglioside complexation; substantial interaction with several relevant lipids; and association between undesirable increases of UC storage in Kupffer cells and UC solubilization.

Interpretation: CDs other than HPβCD identified here may provide disease amelioration without ototoxicity and merit long-term treatment studies. While direct interactions of CD-UC are thought central to the mechanism of correction, the data show that this does not strictly correlate with complexation ability and suggest interactions with other NPC disease-relevant substrates should be considered.

Introduction
Niemann–Pick type C (NPC) disease is a multiorgan storage disorder characterized by lysosomal accumulation of unesterified cholesterol (UC) and other lipids. Central nervous system (CNS) neurons widely display polymembranous cytoplasmic storage bodies with intracellular accumulation of GM2 and GM3 gangliosides in addition to UC. Patients exhibit progressive neurological decline. Mutations of the NPC1 (~ 95% of patients) or
NPC2 gene result in identical disease phenotype.\(^1\) The two encoded proteins, transmembrane NPC1 and soluble luminal NPC2, are thought to interact with UC and/or other lipids in a coordinated fashion to facilitate their egress from late endosomal/lysosomal (LE/LY) compartments.\(^2\)

Therapeutic strategies for NPC disease have included pharmacologic inhibition of substrate accumulation, increasing functionality of defective proteins, and targeting downstream sequelae such as inflammation and oxidative stress.\(^3\) The most efficacious therapy to date has been 2-hydroxypropyl-\(\beta\)-cyclodextrin (HP/\(\beta\)CD) which, following even subcutaneous administration to NPC1- or NPC2-deficient mice, delays clinical onset, extends lifespan, and reduces UC and glycolipid accumulation within the CNS and other organs.\(^4,7\)

Several mechanisms by which therapeutic correction is achieved have been proposed,\(^8,9\) but the predominant view is that cyclodextrins (CDs) directly replace the function of NPC proteins within LE/LY compartments.\(^10\) Supporting this idea, HP/\(\beta\)CD treatment is also found efficacious in mice deficient in both NPC proteins, but not in other diseases with functional NPC proteins and secondary lysosomal storage of cholesterol.\(^4,10\) Exactly how CD acts to emulate NPC protein function or otherwise mediate CNS correction remains unclear.

CDs form host–guest complexes with a wide range of compounds and are commonly used as excipients. These enzyme-modified starch derivatives are cyclic oligosaccharides toroid in shape with a hydrophobic inner cavity and hydrophilic exterior. There are three common “parent” types, \(\alpha\), \(\beta\), and \(\gamma\), composed of 6, 7, and 8 glucose units with increasing inner cavity diameter, respectively. Chemical derivatization of parent CDs is used to change solubility profiles, complexation properties, biodegradability, and toxicity.\(^11,12\) Nearly, all therapy-related studies on NPC animal models have used HP/\(\beta\)CD, a \(\beta\)CD derivatized with hydroxypropyl side groups, yet have paid little attention to how different degrees of substitution (DS), that is, the number of hydroxypropyl groups per CD molecule, might affect efficacy.\(^6,13\) Moreover, the potential efficaciousness of any other CD has been rarely investigated\(^10,14\) and since studies show that HP/\(\beta\)CD is ototoxic,\(^15–17\) identification of safer alternate CDs is greatly needed.

We administered nine different CDs to \(Npc1^{+/−}\) and wild-type (Wt) mice, and evaluated reduction of UC and gangliosides, and ototoxicity apparent through auditory brainstem responses (ABRs). To examine whether therapeutic efficacy related to ability of CD to interact with UC and possibly other accumulating lipids, we conducted parallel complexation assays with the same lots of CDs and UC, GM2 and GM3 gangliosides, and other lipids relevant to NPC disease: glucosylceramide, lactosylceramide, sphingosine, oleic acid, bis(monoacylglycerol) phosphate (BMP), and 24(S)- and 27-hydroxycholesterols (24(S)-HC; 27-HC). This is the first time a direct comparison of in vivo efficacy on disease correction and in vitro solubilization of lipids has been performed for a panel of CDs. Indeed, even just broad comparative complexation studies on many of the CDs and substrates examined here is limited. With the exception of oleic acid, posited to be a substrate of NPC1,\(^16\) all lipids we evaluated are elevated in NPC disease\(^1,19\) and viable candidates for consequential interaction with CD. Sphingosine, glucosyl-, and lactosyl-ceramide are precursors for ganglioside synthesis, and sphingosine accumulation may be an initiating factor in the NPC disease cascade.\(^20\) BMP, enriched in internal LE membranes, can modulate cholesterol homeostasis and sphingolipid metabolism.\(^21,22\) Finally, 24(S)- and 27-HC traffic out of and into the brain,\(^23,24\) and influence CNS cholesterol homeostasis,\(^25\) providing a potential means to impact neuronal storage from outside the CNS, in light of limited CNS entry of peripherally administered CD.

As CD could also promote membrane interactions, similar to what is observed for NPC2,\(^2\) and thereby facilitate efflux of lysosomal UC,\(^26\) we also evaluated each CD’s ability to elicit membrane–membrane aggregation. We found distinct differences among CDs in both efficacy and ototoxicity, including identification of efficacious CDs with reduced ototoxicity, and evidence of substantial and differential complexation with several substrates that could contribute to the outcome of CD-mediated therapy.

**Materials and Methods**

**Animals and treatments**

BALB/cNctr-\(Npc1^{m1N}\)/J heterozygote mice were bred to generate Wt and homozygous affected (\(Npc1^{−/−}\)) progeny and genotyped.\(^27\) From 7–21 days of age, mice were given subcutaneous injections every other day of 2.87 mmol/kg body weight of CD (from a 0.143 mol/L solution) or water alone (vehicle). This dosage corresponds to 4000 mg/kg for HP/\(\beta\)CD (Sigma H107, St. Louis, MO), repeatedly shown to be efficacious.\(^4,5\) Additional cyclodextrins included: three other HP/\(\beta\)CD products differing in manufacturer and DS; 2-hydroxypropyl-\(\alpha\)-CD (HP\(\alpha\)CD); 2-hydroxypropyl-\(\gamma\)-CD (HP\(\gamma\)CD); sulfobutyler-\(\beta\)-CD (SBE\(\beta\)CD); sulfobutyler-\(\alpha\)-CD (SBE\(\alpha\)CD); and sulfobutyler-\(\gamma\)-CD (SBE\(\gamma\)CD) (Table 1). At 3 weeks of age, tissues were collected for analyses as described in Data S1.

Wt mice used for ABR recordings were given weekly subcutaneous injections of 5.74 mmol/kg body weight of CD (from a 0.286 mol/L solution) or water alone starting...
at 8 weeks of age. All animal procedures were carried out according to guidelines approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Tissue staining for ganglioside and unesterified cholesterol

Vibratome sections were stained using immunohistochemistry (IHC) to detect GM2 and GM3 gangliosides, and using fluorescent filipin to detect UC essentially as described28 (Data S1). Widefield digital images were acquired on an Olympus AX70 microscope equipped with a CCD camera (MagnaFire, Optronics). Confocal images were acquired using a Zeiss 510 Meta DuoV2 laser scanning microscope with a 63× (NA 1.4) objective.

Scoring changes in neuronal accumulation of unesterified cholesterol and gangliosides

Three observers blinded to genotype and treatment independently scored representative coronal tissue sections of brain stained for UC, GM2, or GM3 ganglioside. Each coded slide was given a rating from 0 to 10 (0 = no accumulation, 10 = highest accumulation) for that substrate. Scoring was performed on the dorsomedialateral neocortex at ~ bregma – 2.00 mm. Each slide contained ≥ 3 coronal sections from one mouse (3–8 mice/treatment/stain, except filipin staining for Trappsol where n = 2 mice). Scoring was performed at 200×. Data were statistically analyzed with the multigroup nonparametric Kruskal–Wallis test and if appropriate, followed by Dunn’s pairwise comparison post hoc test (P < 0.05).

Biochemical analysis of gangliosides by TLC

Total lipids were extracted from cerebral hemispheres in chloroform–methanol–20% water29, and gangliosides were further separated and quantified as in previous studies.17 Tissue from at least two animals per treatment was analyzed.

Auditory brainstem responses

ABRs elicited by clicks monaurally presented at seven different sound pressure levels (SPLs) were used as an electrophysiological index of hearing thresholds in Wt mice administered different CDs and were assessed at 8, 12, and ~28 weeks of age.

Substrate solubilization by CDs

Biochemical determinations of substrate complexation by CDs were performed in at least one of three ways for each substrate. The first was a mobility shift technique where the target analyte’s migration in a high-performance liquid chromatography (HPLC) chromatogram with increasing amounts of CD was recorded, and the data used to calculate a binding constant.30 The second method also measured mobility shifts, but using capillary electrophoresis and derived an apparent binding constant based on that described.31,32 Wherever possible, traditional phase solubility studies were conducted to obtain solubility isotherms and a binding constant essentially as described.33 The methodology employed for each substrate is indicated in Table 2 and details of the techniques are given in Data S1.
Assay of membrane-membrane interactions by different CDs

Large unilamellar vesicles (LUV) composed of 100 mol % egg phosphatidyl choline (EPC) were mixed with increasing concentrations of each CD, and absorbance at 350 nm (light scattering) was monitored (Versamax microplate reader; Molecular Devices, Sunnyvale, CA) over a 30 min period to assess membrane aggregation.26

Results

Two-week administration of different CDs to \(\text{Npc1}^{-/-}\) mice results in varied cholesterol and ganglioside reduction in the brain

At 3 weeks, UC accumulation in the brain of untreated \(\text{Npc1}^{-/-}\) mice (detectable as fluorescent filipin-positive puncta in neurons, predominantly perikaryal) was differentially reduced by the nine CDs tested. HP/βCD, HP/γCD, and SBE/CD all showed comparable effective reduction of UC storage in the cerebral cortex (Fig. 1), and the four commercial HP/βCDs showed indistinguishable results (Fig. S1). SBE/CD was less effective while αCD-treated matched untreated \(\text{Npc1}^{-/-}\) mice (Fig. 1). These observations were validated by blind scoring of stained tissue sections (Fig. 2A) which, versus untreated mice, showed statistically significant differences only for the HP/βCDs and γCDs. SBE/CD gave intermediate scores, which were not significantly different from either effective CDs or the ineffective αCDs.

In analogous evaluations of reduction of GM2 and GM3 ganglioside accumulation in cortical neurons, different CDs showed a relative efficacy which resembled, but not exactly matched, that for UC. For GM2 (Figs. 1, 2B), very effective statistically significant reduction was again observed with HP/βCD and HP/γCD, but SBE/CD was less effective and not statistically different from vehicle. SBE/CD and αCDs were indistinguishable from vehicle and significantly different from all HP/βCD’s and HP/γCD. The four different brands of HP/βCDs tested (Figs S1, 2B) were all effective and not statistically different from one another. The relative effectiveness of CDs to reduce neocortical GM3 (Figs. 1, 2C and S1) essentially paralleled that seen for UC, again including no significant difference between the different HP/βCDs.

Quantitative biochemical determinations of ganglioside content in cerebral homogenates (Fig. 3) showed trends that largely agreed with immunohistochemical data. For GM2, again all HP/βCD brands and HP/γCD produced values close to that in Wt mice, while αCD values were not lower than untreated \(\text{Npc1}^{-/-}\) mice. SBE/CD also showed effectiveness but more so by thin layer chromatography (TLC) measures than by immunostaining and similarly, SBE/CD showed levels by TLC midway between Wt and untreated \(\text{Npc1}^{-/-}\) while immunostaining scoring was close to untreated mice. For GM3, TLC data paralleled immunostaining patterns: all HP/βCDs and γCDs showed highly effective reduction; SBE/CD exhibited intermediate reduction; and αCDs were indistinguishable from untreated mice.

Table 2. Binding constants (M\(^{-1}\)) for CD interaction with Niemann-Pick type C disease related compounds: unesterified cholesterol, brain-relevant oxysterols, glucosylceramide, lactosylceramide, BMP, sphingosine, and oleic acid.

| Cyclodextrin | UC | GM2 ganglioside | GM3 ganglioside | 24(S)-HC | 27-HC | Gluc-cer | Lac-cer | BMP | Sphingosine | Oleic Acid |
|-------------|----|----------------|----------------|---------|-------|---------|--------|-----|-------------|-----------|
| MβCD | 7800 ± 110 | 2410 ± 250 | 1650 ± 170 | 400 ± 41 | 980 ± 18 | 85 | 1 | 33 | 70 | 1520 ± 25 |
| HPβCD (Sigma) | 4072 ± 22 | 1 | 1 | 117 ± 22 | 480 ± 14 | 1 | 18 | 3 | 215 ± 11 |
| Trappol | 3250 ± 80 | ND | ND | 110 ± 20 | 455 ± 11 | 1 | ND | 13 | 3 | 220 ± 21 |
| Kleptose HPB | 4400 ± 66 | 1 | 1 | 98 ± 36 | 462 ± 8 | 1 | ND | 15 | 5 | 245 ± 18 |
| Kleptose HP | 3050 ± 52 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| HPγCD | 31 ± 12 | 1 | 1 | 13 | 101 ± 3 | 5 | 1 | 7 | 5 | 22 ± 8 |
| SBE/CD | 25 ± 8 | 1; 14 ± 2 | 26 ± 3 | 25 ± 8 | 24 | 1 | ND | 5 | 14 | 13 ± 3 |
| SBE/γCD | 770 ± 29 | 10 ± 2 | 61 ± 9 | 70 ± 29 | 128 ± 11 | 5 | 1 | 3 | 18 | 62 ± 7 |
| HPS/CD | 105 ± 10 | 60 ± 18; | 36 ± 4 | 14 ± 3 | 11 | 10 | 1 | 8 | 8 | 44 ± 11 |
| SBEαCD | 80 ± 13 | 75 ± 13; | 110 ± 17 | 7 | 9 | 5 | ND | 6 | 11 | 36 ± 9 |
| Method used | 1 | 3 | 3 | 1 | 1 | 2 | 2 | 2 | 2 | 1 |

Values reported are average ± SD.

UC, Unesterified cholesterol; Gluc-cer, glucosylceramide; Lac-cer, lactosylceramide; BMP, Bis (monoacyl-glycerol) phosphate; ND, Not done.

1Below limit of detection.

2Methods used for determination were: (1) solubility isotherm; (2) migration shift by high performance liquid chromatography; and (3) migration shift by capillary electrophoresis.
Figure 1. UC, and GM2 and GM3 ganglioside accumulation in the brain cells of 3-week-old mice treated with different CDs. Top row: Sample fluorescence photomicrographs of dorsal neocortex from untreated Wt mouse (A), and CD-treated (B–G) and untreated (H) Npc1<sup>−/−</sup> mice, stained with filipin to detect UC. Virtually, all neurons in untreated Npc1<sup>−/−</sup> mice show positive cytoplasmic staining of UC (white spots) (H), whereas those in Wt mice are negative (A). Note that HPβCD (Sigma) (B), HPγCD (C), and SBEγCD (D) all show highly effective reduction in UC storage, while some UC remains with SBEβCD treatment (E). HPαCD (F) and SBEαCD (G) show UC storage grossly equivalent to untreated mice (H).

Middle row: Sample brightfield photomicrographs of dorsal neocortex stained by immunoperoxidase to detect GM2 ganglioside. Dark brown puncta of GM2 immunoreactivity are evident throughout dorsal neocortical neurons in untreated Npc1<sup>−/−</sup> (H) in contrast to Wt (A) mice. The most effective reduction of GM2 in Npc1<sup>−/−</sup> mouse is seen with HPβCD (B) and HPγCD (C). Noticeably more remaining GM2 is evident in Npc1<sup>−/−</sup> mouse treated with SBEγCD (D), and substantially more with SBEβCD and αCD treatments (E–G) which appear equivalent to untreated Npc1<sup>−/−</sup> mouse.

Bottom row: Sample bright-field photomicrographs of immunoperoxidase stained dorsal neocortex to detect GM3. Dark brown puncta of GM3 immunoreactivity are evident in neurons of untreated Npc1<sup>−/−</sup> mouse (H), though less abundant than GM2, and absent in Wt mouse cortex (A). The relative efficacy of different CDs to reduce GM3 ganglioside parallels UC reduction: HPβCD (B), HPγCD (C), and SBEγCD (D) are nearly indistinguishable from Wt (A); SBEβCD (E) shows an intermediate impact; and αCDs (F–G) show no appreciable reduction. Wt panels for GM2 and GM3 staining are split: Nissl counterstain in left half reveals cortical layers, marked by roman numerals. Scale bars = 50 μm.
Considering UC and ganglioside data collectively, the overall order of efficacy was: all HPβCDs and HPγCD ≥ SBEγCD > SBEβCD > HPαCD and SBEαCD.

Two-week administration of different CDs results in differential changes in cholesterol accumulation in liver

Filipin staining of liver from untreated Npc1+/−/− mice revealed abundant accumulation of UC in hepatocytes and liver macrophages (Kupffer cells). HPβCD and HPγCD showed the greatest clearance of hepatocytic UC storage, followed by SBEβCD and then SBEγCD, while αCDs showed no improvement (Fig. 4). However, all CD treatments led to increased UC accumulation in macrophages in Npc1+/−/− mice, especially with SBEβCD, secondly HPβCD, and least with the αCDs. CD-injected Wt mice also developed striking filipin-positive macrophages with similar relative order of impact seen in Npc1+/−/− mice (Fig. 4).

Ototoxic effects of different CDs as measured by ABRs

ABRs were used as an electrophysiological index of hearing thresholds in Wt mice. Although vehicle-treated mice had thresholds near 40 dB SPL consistent with normal hearing, mice treated with HPβCD had thresholds ≥100 dB SPL, indicative of extreme hearing loss (Fig. 5A, B). Nearly half the mice treated with HPγCD had a threshold at 88 dB SPL, while all other groups (SBEβCD, SBEγCD, HPαCD) had normal thresholds. Importantly, these trends persisted across the three time points span-
In vitro interaction of NPC-relevant substrates with CDs

To determine whether efficacy of different CDs related to their capacity to interact with relevant substrates, in vitro assays were carried out utilizing the same lots of CDs used in vivo. Methyl-β-CD (MβCD), known for its high cholesterol solubilization, was included as a positive standard. In phase-solubility isotherm studies with UC, MβCD showed the highest binding constant (K; equivalent to equilibrium constant) of 7800 ± 110 M⁻¹, and all HP/βCDs were closest to this with Ks between 3050 and 4400 M⁻¹. Ks_{SBE,βCD} was notably lower (770 ± 29 M⁻¹), and the γCDs and αCDs about another order of magnitude lower (Table 2). Complexation values (solubilization at a given concentration; Table 3) followed a similar pattern: MβCD > HP/βCDs (all with comparable values) >> SBE/γCD > SBE/βCD > HP/γCD and αCDs. Thus, results with the βCDs and αCDs trended toward a positive correlation with degree of UC correction in brain, but γCDs particularly HP/γCD, performed better in vivo than the complexation data might predict.

Using the capillary electrophoresis mobility assay, CDs showed either no detectable interaction with ganglioside or Ks ≤ 110 M⁻¹, except MβCD with a K ~ 2000 M⁻¹. K values and their relative order in magnitude for different CDs were similar for both gangliosides, with αCDs > βCDs > γCDs given equivalent side groups (Table 2). Furthermore, SBE-CDs had higher Ks than equivalent parent HP-CDs. The data suggested an inverse relationship between K and efficacy in ganglioside reduction, especially for GM2: the most efficacious, HP/γCDs (all with comparable values) and HP/βCDs trended toward a positive correlation with degree of UC correction in brain, but γCDs particularly HP/γCD, performed better in vivo than the complexation data might predict.

Solubility isotherm studies of the oxysterols showed K values about 10-fold lower for 27-HC than UC, and still lower for 24(S)-HC (Table 2). However, the order of relative magnitude for both oxysterols considerably resembled that seen for with UC: MβCD > HP/βCDs (all brands similar) > SBE/βCD > γCDs > αCDs. Complexation values (Table 3) for 24(S)-HC were highest for MβCD, then HP/βCD (~ 0.02 mol/mol), HP/γCD and SBE-CDs (0.0007–0.0011 mol/mol), and αCDs (0.0001–0.0003 mol/mol), an order compatible with relative UC reduction in vivo, but differences between the latter two groups in complexation were small compared to UC reduction. Relative CD complexation with 27-HC was quite different, with all HP-CDs showing similar high values. Notably, HP/βCD solubilization of 24(S)-HC and 27-HC was equal.
Alternative Cyclodextrins for Treating NPC Disease

C. D. Davidson et al.

Npc1<sup>−/−</sup> filipin Wt

A Untreated

B HP1/CD

C HP1/CD

D SBE<sub>1</sub>/CD

E SBE<sub>1</sub>/CD

F HP<sub>6</sub>/CD

G SBE<sub>6</sub>/CD

Npc1<sup>−/−</sup> CD68 filipin Wt
Figure 4. UC accumulation in liver of mice treated with different CDs. First two columns: (A) Filipin labeling of liver from untreated Npc1<sup>−/−</sup> mice revealed widespread accumulation of UC in hepatocytes and Kupffer cells while liver from untreated Wt mice exhibited only diffuse filipin labeling. (B–G) Npc1<sup>−/−</sup> mice treated with HPβCD, HPγCD, and SBEγCD showed UC reduction within hepatocytes (B, C, D), while SBEβCD, HPαCD, and SBEαCD treatments showed little to no difference from untreated Npc1<sup>−/−</sup> mice (E, F, G vs. A). Administration of all CDs to Wt, as well as disease mice, resulted in elevated UC accumulation in presumptive liver macrophages (Kupffer cells), but hepatocytes remained filipin-negative in Wt mice. Note that the accumulation appeared less in Wt than in disease mice, but the order of impact by different CDs was similar for the two, for example, in both cases, SBEβCD showed the highest and HPβCD the second highest accumulation in Kupffer cells. Second two columns: Confocal images of liver sections double-labeled with anti-CD68 (magenta), to unambiguously delineate lysosomal membranes of Kupffer cells, and filipin (green). (A) CD68<sup>+</sup> Kupffer cells exhibited conspicuous vesicular/vacuolar-like filipin<sup>+</sup> labeling in untreated Npc1<sup>−/−</sup> but not Wt mice. (B–G) CDs tested in Npc1<sup>−/−</sup> instigated varying degrees of increased UC accumulation in Kupffer cells. Wt mice administered different CDs also produced some UC accumulation specifically within Kupffer cells. Scale bars = 50 μm (first two columns), 5 μm (second two columns).

Figure 5. Ototoxicity of different CDs as assessed by auditory brainstem responses (ABR) recordings at 12 weeks of age. (A) Representative ABR recordings from Wt mice administered different CDs are depicted. Several stereotypical ABR waveform components are evident, especially at the higher SPLs tested. Differences in waveform morphologies can be attributed to individual differences across mice and to slight variations in the placement of the subcutaneous needle electrodes. Waveforms obtained at threshold are plotted in red. HPβCD and HPγCD traces show pronounced hearing loss while SBEγCD, SBEβCD, and HPαCD traces demonstrate hearing thresholds equivalent to those of vehicle-treated mice (~40 dB). (B) Plot of hearing thresholds for individual mice reveals minute variability across mice treated with a particular CD with the exception of HPγCD, in which hearing thresholds were more variable. (C) Two-tailed t-tests comparing mean thresholds for each pair of treatment groups revealed statistically significant differences in threshold only between HPβCD or HPγCD and all other treatment groups.
Table 3. Complexation values of CDs with Niemann-Pick type C disease related compounds: unesterified cholesterol, brain-relevant oxysterols, glucosylceramide, lactosylceramide, BMP, sphingosine, and oleic acid.

| Cyclodextrin       | UC/CD\(^a\) | UC/CD\(^b\) | 24(S)-HC/CD\(^c\) | 27-HC/CD\(^d\) | Gluc- cer/CD\(^e\) | Lac- cer/CD\(^f\) | BMP/CD\(^g\) | Sphingosine/CD\(^h\) | Oleic Acid/CD\(^i\) |
|-------------------|-------------|-------------|-------------------|----------------|-----------------|-----------------|-------------|----------------------|---------------------|
| MjCD              | 0.2376      | ND          | 0.0852            | 0.1770         | 5               | 5               | 0.0019      | 0.0067               | 1.3222              | 0.1140              |
| HP\(^\gamma\)CD (Sigma) | 0.0607      | 0.020       | 0.0229            | 0.0402         | 5               | 5               | 0.0229      | 0.3263               | 0.0603              |
| Trappsol          | 0.0805      | 0.021, 0.023| 0.0190            | 0.0360         | ND              | ND              | ND          | ND                   | ND                  |
| Kleptose HPB      | 0.0610      | 0.020       | 0.0177            | 0.0410         | ND              | ND              | ND          | ND                   | ND                  |
| Kleptose HP       | 0.0551      | 0.024       | ND                | ND             | ND              | ND              | ND          | ND                   | ND                  |
| HP\(^\gamma\)CD   | 0.0005      | ND          | 0.0007            | 0.0621         | 5               | 5               | 0.0049      | 0.2059               | 0.0886              |
| SBE\(^\gamma\)CD  | 0.0017      | ND          | 0.0009            | 0.0004         | 5               | ND              | ND          | 0.3929               | 0.0167              |
| SBE\(^\beta\)CD   | 0.0058      | ND          | 0.0011            | 0.0063         | 5               | ND              | 5           | 1.5889               | 0.0123              |
| HP\(^\gamma\)CD   | 0.0004      | ND          | 0.0003            | 0.0352         | 5               | 5               | 0.0104      | ND                   | 0.0384              |
| SBE\(^\alpha\)CD  | 0.0002      | ND          | 0.0001            | 0.0031         | ND              | ND              | ND          | 0.2709               | 0.0260              |

UC, unesterified cholesterol; Gluc-cer, glucosylceramide; Lac-cer, lactosylceramide; BMP, Bis (monoacyl-glycerol) phosphate; ND, Not done.

1Values reported are expressed as mol/mol and were determined from phase solubility isotherms.
2Measurements taken with 250 mg CD/mL H\(2\)O.
3Measurements taken with 50 mg CD/mL H\(2\)O.
4Measurements taken with 10 mg CD/mL H\(2\)O.
5Below limit of detection.

Evidence of stable interaction of the CDs used in vivo with the glycolipids, glucosylceramide, or lactosylceramide, was only detectable for glucosylceramide and limited to HP\(^\gamma\)CD, SBE\(^\beta\)CD, and \(\alpha\)CDs, which showed low Ks (5–10 M\(^{-1}\)) compared to 85 M\(^{-1}\) with MjCD in HPLC migration shift assays (Table 2). Sphingosine, showed an unusually high degree of complexation in phase solubility assays (Table 3) with MjCD and SBE\(^\beta\)CD (~ 1.6 mol/mol) and ~ 4- to 8-fold less with other CDs. K values from shift assays (Table 2) ranged from 3 to 18 M\(^{-1}\), other than for MjCD (70 M\(^{-1}\)). K values for BMP had a similar range, with HP/CDs the highest second to Mj/CD (Table 2). Phase solubility complexation values for BMP with the HP-CDs were similar to or greater than with Mj/CD (Table 3). Lastly, complexation of oleic acid was greater than all other substrates (UC, oxysterols, ceramides, BMP) assayed with 50 mg/mL CD, except for 27-HC:Mj/CD (Table 3). Following Mj/CD, the highest complexation and Ks (Table 2) were obtained with the HP/CDs and HP:CD. HP-CDs gave higher values than equivalent parent CDs with SBE substitutions.

**Membrane interaction potential varies among CD derivatives**

Using assays to quantify aggregation of phosphatidylcholine LUVs, we found Mj/CD and all HP:CD brands produced the greatest aggregation (Fig. 6), followed by HP:CD \(\approx\) SBE\(^\alpha\)CD > SBE\(^\gamma\)CD > HP\(^\gamma\)CD \(\approx\) SBE\(^\beta\)CD. Only Mj/CD and HP/CD values were statistically significantly different from control. Thus, the relative degree of membrane aggregation trended directly with reduction in neuronal UC and ganglioside accumulation obtained for the \(\beta\)- and \(\gamma\)-CDs but not the \(\alpha\)CDs.

**Discussion**

These studies yield several important findings. One, in evaluating the most extensive variety of CDs to date for UC and ganglioside reduction in the brain of Npc1\(^{-/-}\) mice, we find HP:CD as well as HP:CD, the primary CD studied for NPC therapy, are most effective. Furthermore, we show for the first time that SBE\(^\gamma\)CD also provides significant improvement, along with lesser improvements by SBE\(^\beta\)CD. Two, we find that HP:CD is one of the most efficacious CDs, with no significant difference among the four commercial HP:CDs, including Kleptose HPB, which supports its continued use in ongoing clinical trials (NCT01747135 and NCT02534844). Three, we further document HP:CD-induced hearing loss and now demonstrate that repeated high-dose administration of two efficacious CDs, SBE\(^\gamma\)CD and SBE\(^\beta\)CD, show no significant ototoxicity, raising important clinical implications for the future. Four, in parallel biochemical studies, we do not find a complete and positive correlation between CD effectiveness in apparent solubilization of cholesterol or ganglioside, or in phospholipid membrane interaction, and ability to reduce the accumulating substrates in the brain. These studies on NPC disease, which for the first time carried out side-by-side comparisons of CDs in vitro and in vivo, show efficacy cannot be fully predicted based on stable interaction in the aqueous phase with a single substrate.

Patient enrollment in a Phase 2/3 clinical trial began in September 2015 and continues use of the Kleptose HPB...
Alternative Cyclodextrins for Treating NPC Disease

C. D. Davidson et al.

(aka VTS-270) brand of HP/βCD, yet there have been few comparative studies on CDs for their efficacy and none for ototoxicity. A recent study found partial rescue of Purkinje cells and modest increase in Npc1<sup>−/−</sup> mouse survival with HP/βCD, but parallel evaluation with HP/γCD was lacking.<sup>14</sup> It also found that HP/βCD was more effective than HP/βCD in correcting molecular and functional abnormalities in induced neural progenitor cells from NPC patients, while we found these CDs indistinguishable in CNS efficacy in mice with treatment beginning at 1 week of age. Whether early intervention could improve long-term outcome remains uncertain and could pose medical complications. The only other comparative report, in this case of 3 CDs, concluded that SBE/βCD but not HP/βCD matched HP/βCD in normalizing cholesterol synthesis in liver and spleen 1 day after subcutaneous injection.<sup>10</sup> While this measure implies correction of UC transport from the lysosome, our direct visualization of UC storage in cells after 2 weeks of treatment clearly showed HP/βCD was more effective than SBE/βCD in the liver and brain, cautioning that even longer term assessments are desirable given the protracted period of patient therapy. Beyond these previously considered CDs, we found SBE/γCD capable of liver and brain improvements though less efficacious than HP/βCD and HP/γCD. Thus, HP-substituted CDs had greater impact on storage reduction than did the equivalent SBE-substituted parent rings. We found that the DS for HP/βCD (4.5–7.0) made no significant difference in reduction of UC or ganglioside accumulation, in line with reports demonstrating no variance in extending Npc1<sup>−/−</sup> mouse survival<sup>6</sup> or reducing total cholesterol in Npc1<sup>−/−</sup> cells.<sup>13</sup>

All β- and γ-CDs produced reduction of UC in hepatocytes, but interestingly led to an increase in vacuolar UC accumulation in Kupffer cells of Npc1<sup>−/−</sup> mice, as previously observed for HP/βCD,<sup>4</sup> and did so in Wt mice as well though to a lesser extent. Thus, given the latter had no deficiency, this suggests that CDs instigated accumulation in the Kupffer cells, not necessarily that a clearance mechanism in principle cannot operate in these cells. This accumulation might result from enhanced sequestration of extracellular CD as a consequence of the enhanced endocytic activity including phagocytosis associated with macrophages. Excessive levels of endocytosed CD in lysosomes, beyond what would typically result in other cell types such as neurons, could then entrap UC and counter its egress from the lysosome in Wt, as well as in Npc1<sup>−/−</sup> mice where egress is already compromised. This situation may be further aggravated by the fact that macrophages are known to significantly internalize exogenous sources of cholesterol through multiple pathways.<sup>34,35</sup> Alternatively, extracellular precomplexed CD:UC might be accumulated by macrophages, though it has been argued that such complexes are absent from circulation after subcutaneous HP/βCD injections.<sup>36</sup> In any case, our findings that HP/βCD and SBE/βCD showed the largest increase in UC within Kupffer cells and the greatest complexity with UC support the idea that macrophage accumulation of UC is linked to endocytosed CD. Future studies to quantify CD uptake by macrophages and investigate functional consequences would be worthwhile.

Reports of HP/βCD-induced ototoxicity with impact on hearing threshold in NPC1 cats and Wt mice<sup>15–17</sup> have been a major concern in moving CD forward as a therapeutic agent. Importantly, we found that HP/γCD which was also therapeutically efficacious, showed significantly less ototoxicity than HP/βCD in Wt mice, and that the equivalent SBE-substituted βCD and γCD, showed no ototoxicity as assessed by ABR measures. While these findings must be confirmed in Npc1<sup>−/−</sup> mice, a comparative study on HP/βCD-induced toxicity by several criteria on Wt and disease mice, indicated Npc1<sup>−/−</sup> mice were more resistant, not less, to toxic effects,<sup>17</sup> suggesting these alternate CDs should not produce greater ototoxicity in Npc1<sup>−/−</sup> mice. It is unclear why HP/βCD causes death of

Figure 6. Membrane–membrane interaction of different CDs. Absolute change in absorbance after 30 min incubation period of 100 µmol/L Large unilamellar vesicles (LUVs) with 1 mmol/L CD provides a measure of LUV aggregation (mean ± SE, N = 3 experiments). A highly significant statistical difference was found among samples (ANOVA, P < 0.0001). Post hoc analysis (Tukey’s multiple comparison test) showed that each of the HP/βCDs and MJ/CD were significantly different from control (LUVs with buffer only) (P < 0.01; asterisks), and there was no significant difference (P > 0.05) among these CDs in pairwise comparisons. While the values for other CDs variably trended above background levels, this aggregation was not found statistically significantly different from control, nor between these other CDs. Similar results were obtained with CDs at 10 µmol/L and 100 µmol/L in three independent experiments (data not shown).
outer hair cells of the cochlea, which underlies the hearing loss, but our observed ototoxicity order of HP/βCD > HP/γCD > HP/ζCD matches that reported for hemolysis and toxicity for other cell types. In addition, SBE/βCD with ~ 7DS, such as we used, showed no hemolysis in contrast to HP/βCD at equivalent concentrations. Correlations between hemolytic activity and degree of cholesterol solubilization have been reported (e.g.,), but neither our UC:CD complexation data, nor data from any other substrate examined, showed clear predictive power for ototoxicity aside from the observation that HP/βCD exhibited both the greatest ototoxicity and UC complexation. Multiple interactions of CD with different hair cell constituents, negative effects at the blood-labyrinth barrier or even disruption of fragile perilymph homeostasis may contribute to toxicity.

Collectively, our therapeutic efficacy and toxicity data suggest that the two effects are to some extent separable and provide a basis for further investigation in Npc1−/− mice. Of particular interest is HP/βCD which showed equivalent efficacy to HP/βCD but reduced ototoxicity and macrophage involvement, and SBE/γCD which elicited even fewer side effects but with some reduction in efficacy.

Given the known affinity of βCDs for cholesterol, the therapeutic action of HP/βCD has been thought to arise from interaction with UC, be it in extracellular/interstitial fluid, at the plasma membrane or within LE/LYs. Indeed, the findings that both UC solubilization and storage reduction was far higher for the βCDs than the ζCDs, and that HP/βCD was better than SBE/βCD in both regards, support this view. On the other hand, γCDs appeared closer to HP/βCD in efficacy but to ζCDs in solubilization. Studies pertaining to CNS entry of CDs are inadequate to assert that differential access to neurons could account for these discrepancies and do not suggest γCDs are more effective than ζCDs in this regard.

Relative reduction of UC storage in the brain by different CDs was also very similar to that seen in hepatocytes, further suggesting that blood–brain barrier (BBB) penetrability was not a discriminatory factor. Should the critical mode of CD’s action occur within LE/LYs, as many contend, it could promote UC egress through direct interactions with membranes. This may occur via diffusion of substrate directly from the membrane into the hydrophobic core of CD or CD may lower the activation energy for desorption from membrane into aqueous phase followed by rapid transfer to an adjacent membrane, that is, movement of accumulated UC from LE/LY multilamellar membranes to the limiting membrane. In fact, we observed a trend for the different β- and γCDs that showed the same order of potency for promoting membrane–membrane interaction (Fig. 6) as observed for reduction of UC storage. Thus, pure aqueous solubilization measurements may not be adequately predictive, but the exceedingly low values found for the γCDs, especially HP/γCD, calls for consideration of yet other critical mechanisms. Studies directly comparing CDs for UC transfer between adjacent bilayer membranes could be insightful.

It is not clear why GM2 and GM3 accumulate in NPC disease or why HP/βCD can counter this. Although speculated, there is no definitive evidence that the NPC proteins directly interact with gangliosides or their metabolic proteins. However, there is evidence of colocalization of gangliosides with UC in LE/LY, particularly striking for GM3, suggesting possible interdependence or association of ganglioside and UC accumulation. We also found relative reduction of GM2, and even more so of GM3, to parallel efficacy of CDs for UC reduction, further arguing a close link between these compounds. These observations are also compatible with the prevailing view that CD mediates correction from within LE/LYs. Additionally, the CDs showed a significant inverse correlation between their Ks for GM2 or GM3 and their reduction of these gangliosides in brain (Fig. S3), suggesting detrimental effects of CD on ganglioside clearance. In fact, we observed a small increase in % GM2 and GM3 gangliosides in Npc1−/− mice treated with ζCDs (which showed the highest Ks). It is conceivable that direct interaction with CDs modulated efficiency of ganglioside catabolism. On the other hand, reduction of ganglioside accumulation in LE/LY may arise indirectly. A recent in vitro study showed that elevated membrane cholesterol can inhibit GM2 activator protein function and efficiency of GM2 hydrolysis. Accordingly, CDs more effective at reducing stored UC should also produce greater GM2 reduction, which is what we found.

From the remaining substrates tested, of note were the traditional phase solubility measurements (Table 3) for sphingosine which included values even greater than 1 mol/mol with M/βCD and SBE/βCD suggesting formation of sphingosine:CD complexes of > 1:1 stoichiometry. The potential therapeutic effects of CD through interaction with sphingosine merit further investigation, as sphingosine is elevated early in NPC disease and has been hypothesized to be an initiating pathogenetic factor for both UC and ganglioside storage.

Of all the substrates evaluated, the relative degree of complexation among CDs with 24(S)-HC arguably best approached a correlation with UC storage reduction though this interaction too was imperfect and cannot explain the efficacy findings alone. The range of solubilization values of oxysterols, 0.0001–0.06 mol:mol CD, which resembled and approached levels seen with UC, is worth noting given the levels of HP/βCD and the much lower concentration of oxysterols than cholesterol found in the circulation. After a single subcutaneous injection...
equal to the one we used, plasma HP/βCD peaks at ~ 1.4 or 3.5 mmol/L, depending on mouse age. In Wt mice, 24(S)-HC and 27-HC are each found at ~ 0.1 μmol/L, respectively, in plasma. Thus, the mol oxysterol:mol CD ratio would be 0.00004, well below even the lowest solubilization capacity we measured. Therefore, while BBB penetration of CDs is limited, neuronal accessibility to these oxysterols which purportedly influence cellular cholesterol homeostasis and are the primary sterols that traverse the BBB B23,24 has the potential to be modulated through peripheral interactions with CDs.

In sum, complexation data showed incomplete correlation with efficacy for UC, an inverse correlation for GM2 and GM3, and the closest to a direct correlation for 24(S)-HC. Substantial solubilization was also obtained for 27-HC, sphingosine, and oleic acid. Potential influences on efficacy through CD interactions, particularly with sphingosine, gangliosides, and the oxysterols, merit further investigation, particularly in an environment that more closely mimics in vivo conditions. It may well be that the sum of CD interactions with several compounds, in turn altering their availability for endogenous molecular interactions, determines efficacy. Such influences could conceivably account for broader subcellular mechanisms that have been implicated in CD-mediated disease amelioration. These mechanisms include induction of lysosomal exocytosis, TFEB-mediated changes in lysosomal biogenesis, and modulation of the autophagic pathway. Rational development of improved CD-based therapeutics can benefit from understanding the key interactions involved, but our empirical findings already demonstrate that efficacy and toxicity are not wholly inseparable, and viable alternatives to HP/βCD are possible. Immediate goals should be long-term efficacy studies of SBE-CDs to fully understand their therapeutic value and validate lack of ototoxicity, with the hope of providing better options for NPC patients and their families.

**Author Contribution**

CDD, SUW, and KD conceived, designed, and analyzed studies. All authors contributed to design of experiments. TS, LSz, JSz, MTV, YF, IP, JSz, LAM participated in interpretation of the data. CDD and KD wrote the manuscript with contributions from YF, LAM, JS, TS, and LSz, and all authors contributed to editing of the manuscript.

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**Conflict of Interest**

SUW is a member of the preclinical SAB for Vtesse, Inc. The other authors have no conflicts.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1. UC and GM2 and GM3 ganglioside accumulation in brain cells of 3-week-old mice treated with different commercial preparations of HPβCDs.
Figure S2. Ototoxicity of different CDs as assessed by ABR recordings.
Figure S3. Correlation between reduction of gangliosides in Npc1−/− cerebral cortex and evidence of stable interaction with different CDs.
Data S1. Supplementary Materials and Methods.