Analytical Characterization of Methyl-β-Cyclodextrin for Pharmacological Activity to Reduce Lysosomal Cholesterol Accumulation in Niemann-Pick Disease Type C1 Cells

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INTRODUCTION

Niemann-Pick disease type C (NPC) is an inherited, progressive neurodegenerative disorder caused by mutations in either the NPC1 or NPC2 gene, each of which encodes two distinct lysosomal cholesterol-binding proteins.1,2 Deficiency in NPC1 or NPC2 protein results in malfunction of intracellular cholesterol trafficking and accumulation of unesterified choleseters in late endosomes/lysosomes.3,4 The clinical manifestations of NPC include hepatosplenomegaly and progressive neurodegeneration, a hallmark of the disease.3 Currently, there is no effective treatment for NPC disease, although a number of agents have shown the therapeutic potential for treatment of NPC, including 2-hydroxypropyl-β-cyclodextrin (HPβCD), an analog of methyl-β-cyclodextrin (MβCD), and miglustat.5–13 Several other treatments for NPC disease are currently being investigated, including histone deacetylase (HDAC) inhibitors and the c-Abl inhibitor, imatinib.14–19

Cyclodextrin showed remarkable in vivo efficacy in reduction of neurodegeneration and increase in life span of npc1−/− mice.20–23 Cyclodextrins are cyclic oligosaccharides consisting of multiple glucopyranose units. Cyclodextrins typically exist as hexamers (α-cyclodextrins), heptamers (β-cyclodextrins), or octamers (γ-cyclodextrins); all are highly water soluble with hydrophobic cavities of different sizes (Fig. 1). Originally used as a Food and Drug Administration-approved excipient for pharmaceutical formulations of lipophilic compounds, HPβCDs fortuitously also show pharmacological activity. Both MβCD and HPβCD have been reported to reduce lysosomal cholesterol accumulation and enlarged lysosomes in NPC patient cells.24–27 HPβCD is currently in clinical trials for the treatment of NPC.28,29 However, the reported efficacy and potency of β-cyclodextrins varied between different laboratories.4,30–34 These differences could

Keywords: lysosomal storage disease, niemann-pick disease type C1, methyl-β-cyclodextrin, mass spectrometry, proteomics
be caused by variations of HPβCD and MβCD preparations from the manufacture processes.

To develop an analytical method for selection of an effective cyclodextrin preparation as a therapeutic agent, we tested three preparations of MβCDs for their profiles with mass spectrometry (MS) analysis, pharmacological activity, and protein expression levels in NPC1 patient cells. We found that average molecular weights and levels of methylation in side chains of MβCD molecules are different in these three preparations obtained from two vendors and each from a different batch. The higher level of methylation is likely to be more efficient at reducing unesterified cholesterol accumulation in lysosomes of NPC1 patient cells. Further proteomic profiling analysis revealed that MβCD ameliorates dysregulated protein expression in NPC1 disease cells, including proteins involving lysosome formation, vesicle expansion, and completion during autophagy, SNARE interactions in vesicular transport, and steroid biosynthesis. These results demonstrate that the combination of mass spectrum profiling, pharmacologic activity on reduction of lysosomal cholesterol accumulation, and the signature of ameliorating abnormal protein expression in NPC1 patient cells is a useful analytic method for characterization and selection of high efficacy cyclodextrin preparations for use as a therapeutic agent.

MATERIALS AND METHODS

Materials

Three different sources of MβCDs were used for the experiments. The first batch of MβCD (MβCD-1) was purchased from MP Biochemicals [Heptakis (2, 6-Di-O-methyl)-β-cyclodextrin, catalog No. 157320, Lot No. M1322]. The second batch of MβCD (MβCD-2) was from Sigma (Methyl-β-cyclodextrin; catalog No. C4555, Lot No. SLBH6893V) and the third batch of MβCD (MβCD-3) was also purchased from MP Biochemicals, but from a different lot number (catalog No. 157320, Lot No. 9355K).

Cells and Cell Culture

NPC1 patient skin fibroblasts (NPC1, ILE106ITHR, GM03123) and human wild-type (WT) fibroblasts (GM05659) were purchased from the Coriell Cell Repository (Camden, NJ). The fibroblasts were cultured in Dulbecco’s modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum.
H₂O₂ and the corresponding ketone product. H₂O₂ is then samples are hydrolyzed by cholesterol esterase to free cho-
esterified cholesterol (free cholesterol) and cholesteryl esters in blood samples and cell lysates. Cholesteryl esters in the
concentrations in samples. It can be used to measure un-
esterified cholesterol (free cholesterol) and cholesteryl esters
Technologies, Carlsbad, CA). This biochemical assay uses an
enzymatic coupled reporting system for detection of cholesterol
Amplex® Red Cholesterol Assay
Total cholesterol in patient cells was measured using the
Amplex® Red cholesterol assay kit (catalog No. A12216; Life
Technologies, Carlsbad, CA). This biochemical assay uses an
enzyme-coupled reporting system for detection of cholesterol
cell nuclei were stained with 100 µL/well of 4 µM ethidium
homodimer (EthD-1; Life Technologies) in DPBS at room
temperature for 30 min. The plates were imaged using an IN
Cell Analyzer 200 cell imaging system (GE Healthcare) with
LysoTracker Red Dye Staining of Cell Lysosome
LysoTracker® dyes consist of a fluorophore linked to a weak
base that concentrate in and stain cellular acidic compart-
ments, including late endosomes and lysosomes. The Lyso-
Tracker dye staining assay has been optimized to visualize
enlarged lysosomes at the proper dye concentration in NPC
patient cells, while control WT cells exhibit minimal stain-
ing. Briefly, cells were seeded in black, clear bottom, tissue
culture-treated 96-well plates (catalog No. L-7528; Life Technologies) in DPBS, followed by incubation at
room temperature for 30 min. After washing twice with DPBS, the plates were fixed with 4% PFA for 30 min at room
temperature and washed twice with DPBS. Nuclear staining
was performed by addition of 100 µL/well, 1 µg/mL Hoechst
33342 (Life Technologies) in DPBS, followed by incubation at
room temperature for 30 min. After washing twice with DPBS, the plates were stored at 4°C until imaging. The image ac-
quisition was carried out in the IN Cell Analyzer 2000 cell
imaging system. The DAPI filter set (excitation = 350 ± 25 nm,
emission = 455 ± 25 nm) and TRITC filter set (excitation = 545 ± 10 nm, emission = 593 ± 10 nm) were used to visualize
Hoechst nuclear staining and LysoTracker red staining,
respectively.
ATP Content Assay for Cell Viability Measurement
An ATP content assay kit (ATPLite; PerkinElmer) was used to
measure cell viability to assess compound cytotoxicity. ATP
is present in all metabolically active cells. The ATP concen-
tration declines rapidly when cell death occurs. This assay
method utilizes a luciferase report system to measure cellular
ATP level that is proportional to viable cell numbers and cell
health conditions. The advantages of this ATP content assay
(10 mg/mL and then diluted in DPBS) at room temperature for
1 h. The plates were stored at 4°C after washing twice with
DPBS before imaging analysis. On the day of the imaging,
were washed twice with Dulbecco’s phosphate-buffered saline
(DPBS; Life Technologies) with Ca²⁺ and Mg²⁺ and fixed with
100 µL/well of 4% paraformaldehyde (PFA; Electron Micro-
scopy Sciences, Hatfield, PA) solution at room temperature for
30 min. After washing twice with DPBS, the cells were stained
with 50 ng/mL filipin solution (freshly dissolved in DMSO at
Filipin Fluorescence Staining of Free Cholesterol
Filipin dye (catalog No. F9765; Sigma) is a polyene mac-
rolide antibiotic and antifungal compound. It has been used as
a profluorescence probe to stain the free cholesterol in cells or
tissues, but not the esterified cholesterol at an appropriate
concentration. Briefly, cells were seeded at 2,000 cells/well
in 100 µL of medium in black, clear bottom, tissue culture-
treated 96-well plates and cultured for 24 h. Compounds dis-
solved in the media were added to cells in the assay plates at 100 µL/well and incubated for another 4 days. The cells were then washed twice with Hank’s Balanced Salt Solution (Life Technologies) and 100 µL/
well of reagent mixture from the Amplex Red cholesterol
assay kit was added. Fluorescence intensity in assay plates
after 1 h of incubation at 37°C was measured at an excitation
wavelength of 560 (±10 nm) and emission wavelength of 590
(±10 nm) in a fluorescence plate reader (Tecan, Durham, NC) or
ViewLux® plate reader (PerkinElmer).

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include high assay sensitivity and one-step addition homogeneous assay format. Briefly, cells were seeded at 2,500 cells/ well in 100 μL medium in white, solid 96-well plates (catalog No. 655083; Greiner Bio-One) and incubated for 24 h. Cells were cultured and treated as described above for the cholesterol assay. After 4 days of incubation, 150 μL/well of ATP content reagent mixture (prepared according to the manufacturer’s instructions) was added to the assay plates, followed by incubation at room temperature for 30 min protected from light. The luminescence signal was determined in the luminescence mode of the ViewLux plate reader.

Data Analysis and Statistics
Image analysis of filipin staining and LysoTracker red staining was conducted using IN Cell Analyzer software (version 3.7.2; GE Healthcare). The Multi-Target Analysis protocol was used for quantification of Hoechst-stained nuclei, LysoTracker red-stained lysosomes, EthD-1-stained nuclei, and filipin-stained unesterified cholesterol. Concentration–response curves were analyzed and IC_{50} values calculated using Prism software (GraphPad, Inc., San Diego). Results in the figures are expressed as means of triplicates ± standard deviations. Unless otherwise stated, an unpaired t-test was used to test for significance, with *P < 0.05, **P < 0.01, and ***P < 0.001.

MS Analysis of MβCD
To identify the average molecular weight and levels of methylation in side chains of MβCD molecules, we carried out the MS analysis. Initially, 0.4 mg of MβCD powder was dissolved in 3 mL of deionized Millipore (Sigma-Aldrich, St. Louis, MO) water as the stock solution (100 μM). An aliquot of the MβCD stock solution was further diluted with 90% methanol to 10 μg/mL (8 μM) for MS analysis. The diluted MβCD solution was directly infused into the API-4000 Mass Spectrometer (Applied Biosystems, Forster City, CA) at a rate of 10 μL/min. The mass spectrometer was used for Q1 scan with positive ion electrospray mode at 5000V and 100V de-clustering potential. The MS scan range was adjusted from m/z 1,200 to 1,450 at a scan rate of 2 s. The data analysis was performed by Analyst 1.5.1 software (Applied Biosystems, Foster City, CA).

Proteomic MS Analysis
Preparation of protein samples for liquid chromatography–tandem mass spectrometry analysis. MS proteomic analysis has been widely used to analyze biological samples and to quantitate protein levels in samples. For example, changes of cellular protein levels can be detected quantitatively in patient cells compared with WT control cells. To further understand the cholesterol reduction effect of MβCD, we carried out a proteomic analysis of NPC1 patient fibroblasts after treatment with MβCD.

NPC1 (GM03123) and WT (GM05659) fibroblasts (1 x 10^6 cells) were seeded into tissue culture-treated T-75 flasks; cells were treated with different sources of MβCDs (250 μM). After 4 days of treatment, cells were collected using TrypLE™Express (Life Technologies) and washed with DPBS twice. Cell pellets were collected by centrifugation at 250 g for 5 min and stored at −80°C before sending to Poochon Scientific for proteomic MS analysis.

Total protein extraction from cells was prepared by the following method. In brief, cell pellets were lysed in 0.4 mL lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2% sodium dodecyl sulfate), followed by sonication. The cell lysate was clarified by centrifugation at 15,000 g for 10 min and supernatant was collected and stored at −80°C for further analysis. The protein concentration of the supernatant was determined by a BCA™ Reducing Reagent compatible assay kit (Thermo Scientific, Rockford, IL).

Equal amounts of protein (130 μg) from each sample were fractioned by separation on NuPAGE 4%–12% Bis–Tris Gel (Life Technologies); 16 gel fractions from each lane representing one sample were treated with dithiothreitol (DTT) for reduction, then iodoacetamide for alkylation, and further digested with trypsin in 25 mM NH_4HCO_3 solution. The digested protein was extracted as described elsewhere. The extracted peptides were dried and reconstituted in 20 μL of 0.1% formic acid before the nanospray liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed.

Nanospray LC-MS/MS analysis. Sixteen tryptic peptide fractions from one cell sample were analyzed sequentially using a Thermo Scientific Q-Exactive™ hybrid Quadrupole-Orbitrap™ Mass Spectrometer equipped with a Thermo Dionex UltiMate™ 3000 RSLCnano System. Tryptic peptide samples were loaded onto a peptide trap cartridge at a flow rate of 5 μL/min. The trapped peptides were eluted onto a reversed-phase 25-cm C18 PicoFrit column (New Objective, Woburn, MA) using a linear gradient of acetonitrile (3%–36%) in 0.1% formic acid. The elution duration was 110 min at a flow rate of 0.3 μL/min. Eluted peptides from the PicoFrit column were ionized and sprayed into the mass spectrometer using a Nanospray Flex™ Ion Source ES071 (Thermo Fisher) under the following settings: spray voltage, 1.6 kV, and capillary temperature, 250°C. The Q-Exactive instrument was operated in the data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 300–2000) were acquired in the
Orbitrap with 70,000 resolution (m/z 200) after the accumulation of ions to a $3 \times 10^6$ target value based on predictive automatic gain control (AGC) from the previous full scan. Dynamic exclusion was set to 20 s. The 12 most intense multiply-charged ions ($z \geq 2$) were sequentially isolated and fragmented in the Axial higher energy collision-induced dissociation (HCD) cell using normalized HCD collision energy at 25% with an AGC target 1e5 and a maxima injection time of 100 ms at 17,500 resolution.

**LC-MS/MS data analysis.** The raw MS files were analyzed using the Thermo Proteome Discoverer™ 1.4.1 platform (Thermo Scientific; Bremen, Germany) for peptide identification and protein assembly. For each cell sample, 16 raw MS files obtained from 16 sequential LCMS analyses were grouped for a single database search against the Human UniProtKB/Swiss-Prot human protein sequence databases (20,608 entries, as of December 20, 2015) based on the SEQUEST and percolator algorithms through the Proteome Discoverer 1.4.1 platform. Carbamidomethylation of cysteines was set as a fixed modification. The minimum peptide length was specified to be five amino acids. The precursor mass tolerance was set to 15 ppm, whereas fragment mass tolerance was set to 0.05 Da. The maximum false peptide discovery rate was specified as 0.01 or 0.05. The resulting Proteome Discoverer Report contains all assembled proteins with peptide sequences and matched spectrum counts. Protein quantification used the normalized spectral abundance factor (NSAF) method to calculate the protein relative abundance.

**Pathway analysis.** The cell functions are executed and regulated by entire sets of proteins (the proteome). The regulation of different cellular functions has been categorized into a number of signaling pathways, such as cell cycle and apoptosis. In each pathway, components are usually named according to their function: ligands, receptors, activating regulators, inhibitory regulators, and effectors. To measure the activation strength of a pathway, proteins classified as ligand, receptors, activating regulators, or inhibitory regulators were grouped and their relative abundances (ppm) were summed. The protein list for all analyzed pathways and processes was obtained from the KEGG pathway database (www.genome.jp/kegg/pathway.html) and their functional annotations were manually confirmed using the UniProtKB protein database, the NCBI protein database, or available publications.

**RESULTS**

**MβCD MS Analysis**

Beta-cyclodextrins are complex molecules with a mixture with various positions and amounts of substitution groups. Hence, the details of cyclodextrins vary between manufacturers and between different batches by the same manufacturer. Variations between preparations of β-cyclodextrins used as therapeutic agents for the treatment of human disease can lead to variations in the efficacy of these treatments. To characterize the molecular profiles of MβCD preparations, we measured the profiles of mass spectrum of these three MβCD preparations (MβCD-1, MβCD-2, and MβCD-3).

Methyl substitutions of the MβCD-1 sample range from 7 (7-Me) as the lowest methyl substitution to 15 (15-Me) as the highest (Fig. 2A). The most abundant methyl-β-cyclodextrin is 10-Me, followed by 11-Me and 9-Me, while the lowest and highest methyl substitutions of MβCD-2 are 8-Me and 17-Me (Fig. 2B). The distribution of methyl-β-cyclodextrins in MβCD-2 is more spread out than the other two preparations. For MβCD-2, the most abundant methyl-β-cyclodextrin is 13-Me, followed by 12-Me and 14-Me. The distribution of methyl-β-cyclodextrins in MβCD-3 seems to be less spread out than MβCD-1 and MβCD-2; the distribution from 13-Me to 18-Me is shown in Figure 2C. Interestingly, the most abundant methyl-β-cyclodextrin is 14-Me, followed by 15-Me and 16-Me, respectively, for the MβCD-3 preparation.

MS response of individual methyl-β-cyclodextrins was assumed to be nearly equal since the size and polarity of the molecules are close to each other. The average exact molecular weights of three MβCDs are determined by these MS analyses, by first measuring the signal height of the sodium adduct molecular ion of each methyl-β-cyclodextrin, then calculating the molecular distribution, and finally determining the molecular weight contribution (component) in Table 1. MβCD-1 has the lowest molecular weight, followed by MβCD-2, while MβCD-3 has the highest molecular weight. These results indicated that all three MβCD preparations are different in the number of substitution groups and average molecular weights.

**Reduction of Lysosomal Cholesterol Accumulation in NPC1 Fibroblasts by MβCD**

Filipin staining of lysosomal unesterified cholesterol in NPC1 patient fibroblasts is used for diagnosis of the disease and for evaluation of therapeutic activity of the compounds. The effect of β-cyclodextrins on decreasing filipin staining and lysosomal cholesterol accumulation in NPC1 cells has been reported previously. To determine if these three MβCD preparations reduce lysosomal cholesterol accumulation, we measured their effect on filipin staining in NPC1 fibroblasts. While the lysosomal filipin staining was significantly increased in NPC1 fibroblasts, treatment with MβCDs reduced filipin staining in NPC1 fibroblasts (Fig. 3A). MβCD-3

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significantly reduced filipin staining with IC$_{50}$ of 19.2 µM. However, much weaker activities were found for MβCD-1 and MβCD-2 (Fig. 3A–C). The maximal inhibitory effect on cholesterol accumulation was 48.1% for MβCD-3 compared with 17.8% for MβCD-1 and 20.9% MβCD-2 (Table 2). Similar results were observed in two other independent experiments. MβCD-1 and -3 were obtained from the same vendor with different lot numbers (suggesting that they were manufactured in different times); nonetheless, their activities were significantly different. These results revealed significant differences in MβCD pharmacological efficacy among three preparations.

We also measured cellular cholesterol levels in NPC1 cells using an Amplex Red cholesterol assay. The Amplex Red cholesterol assay is a biochemical assay utilizing an enzyme-coupled reporting system to quantitatively measure both free cholesterol and cholesteryl esters. MβCD-3 reduced the cellular unesterified cholesterol levels in NPC1 fibroblasts in a concentration-dependent manner, similar to previous reports. The IC$_{50}$ value of MβCD-3 for reduction of cellular cholesterol level was 14.4 µM. Similarly, as seen in the filipin staining experiment, efficacies of MβCD-1 and MβCD-2 were much weaker compared with that of MβCD-3. The maximum inhibitory effect was 46.0% for MβCD-3, but
was only 15.2% for MβCD-1 and 19.5% for MβCD-2 (Fig. 4A, B, and Table 2). We observed similar results in two other independent experiments. We also determined cytotoxicity effects of MβCDs using an ATP content assay, but did not observe any toxic effect of these three preparations at the highest compound concentration of 300 μM (Fig. 4C).

Decrease of Enlarged Lysosomes in NPC1 Fibroblasts by MβCD

The lysosomes are usually enlarged in NPC1 patient cells due to accumulation of cholesterols that can be detected using LysoTracker dye staining. To further determine the pharmacological activity of three MβCD preparations, we measured LysoTracker dye staining in NPC1 fibroblasts...
after MβCD treatment. LysoTracker dye staining was significantly increased in NPC1 cells, indicating enlarged lysosomes in these cells when compared with WT cells. MβCD-3 significantly reduced LysoTracker dye staining in NPC1 fibroblasts with an IC₅₀ of 28 μM and the maximum inhibitory effect of 36.8% (Fig. 5 and Table 2). In striking contrast, there was no significant decrease in lysosome size of cells treated with MβCD-1 and MβCD-2. The maximum inhibitory effects for MβCD-1 and MβCD-2 were 12.6% and 4.5%, respectively. Similar results were observed in two other independent experiments. All these results showed that MβCD-3 was much more potent than MβCD-1 and MβCD-2 for reduction of enlarged lysosomes in NPC1 cells, consistent with the observed differences in reduction of cholesterol accumulation in NPC1 cells from previous tests.

Proteomic Signature of Effect of MβCD Treatment in NPC1 Fibroblasts Analyzed by Proteomic Profiling

Only a few gene or protein profiling studies for NPC1 disease have been reported to date.⁴⁰–⁴⁵ To further examine the activity of MβCD on dysregulated protein expression in NPC1 cells, we carried out a proteomic analysis of protein expression in the NPC1 fibroblasts after treatment with MβCD. We identified a panel of 19 proteins that were dysregulated in NPC1 fibroblasts (Fig. 6). These proteins are involved in lysosomal vesicle trafficking, which includes lysosome formation, vesicle expansion, and completion during autophagy, SNARE (soluble NSF attachment protein receptor) interactions in vesicular transport, and steroid biosynthesis. As shown in Figure 6, NPC1 protein was downregulated, while NPC2 protein (Fig. 6B) and lysosomal protein transmembrane 4 alpha (LAPTM4A) were upregulated in NPC1 cells. Unconventional SNARE in the ER 1 (USE1) (Fig. 6C) and the vesicle-associated membrane protein 7 (VAMP7) (Fig. 6D) involved in SNARE interactions in vesicular transport were also dysregulated in the opposite direction in NPC1 fibroblasts. The GABA (A) receptor-associated protein (GABARAP) (Fig. 6E) and GABA (A) receptor-associated protein-like 1 (GABARAPL1) and 2 (GABARAPL2) involved in vesicle expansion and completion

### Table 2. Efficacy of Three Different Methyl-β-Cyclodextrin Preparations

| Assay          | MβCD-1 (μM) | Efficacy (%) | MβCD-2 (μM) | Efficacy (%) | MβCD-3 (μM) | Efficacy (%) |
|----------------|-------------|--------------|-------------|--------------|-------------|--------------|
| Filipin        | 5.9         | 17.8         | 2.8         | 20.9         | 19.2        | 48.1         |
| LysoTracker®   | NA          | 12.6         | NA          | 4.50         | 28.3        | 36.8         |
| Amplex Red®    | 2.5         | 15.2         | 3.5         | 19.5         | 14.4        | 46.0         |

**Fig. 3.** Effects of different sources of MβCDs on reducing cholesterol accumulation in NPC1 fibroblasts. NPC1 patient skin fibroblasts (GM03123) and WT control (GM05659) were untreated or treated with MβCD (0.4–300 μM) for 4 days; filipin staining was then performed. (A) Images of filipin staining on NPC1 fibroblasts. Treatment with 300, 11 μM of MβCD-3 significantly reduced cholesterol accumulation in NPC1 patient skin fibroblasts, while the other two batches of MβCDs (MβCD-1 and MβCD-2) showed much weaker effects on cholesterol accumulation in NPC1 patient fibroblasts. Filipin (green) stains the intracellular cholesterol-laden domains, and EthD-1 (red) stains nuclei. (B) Treatment with MβCD-3 (300 μM) significantly reduced cholesterol accumulation in the NPC1 patient fibroblast compared with the other two batches of MβCDs (MβCD-1 and MβCD-2). (C) Dose–response curve of different sources of MβCDs on cholesterol accumulation in NPC1 patient fibroblasts. EthD-1, ethidium homodimer; NPC1, Niemann-Pick disease type C1; WT, wild-type.
during autophagy were all upregulated in NPC1 cells. Several key enzymes involved in steroid biosynthesis, such as NAD (P)-dependent steroid dehydrogenase-like (NSDHL) and 24-dehydrocholesterol reductase (DHCR24) were also overexpressed in NPC1 cells (Fig. 6A, F, and G). The expression changes of these 19 proteins in NPC1 cells were also confirmed in a repeat proteomic MS experiment.

After treatment of NPC1 cells with MβCD-3 for 4 days, the increased NPC2 level was significantly reduced, while there was no significant effect on the decreased NPC1 level (Fig. 6B). These results are consistent with previous reports that NPC1 protein level was not affected by treatment with cyclodextrin, although cyclodextrin reduced lysosomal cholesterol accumulation.27 The decrease in elevated NPC2 level in NPC1 cells could result from the MβCD reduction of cholesterol accumulation and improvement of cholesterol trafficking. The dysregulated USE1 (Fig. 6C), VAMP7 (Fig. 6D), GABARAP (Fig. 6E), NSDHL (Fig. 6F), and DHCR24 (Fig. 6G) were also recovered after MβCD treatments. Similar to the observed pharmacological activities, different batches of MβCDs showed varied effectiveness on the return to normal expression levels of these 19 dysregulated proteins. MβCD-3 exhibited a much better recovery in NPC1 fibroblasts when compared with MβCD-1 and -2, consistent with the observation of the highest reduction of unesterified cholesterol level by MβCD-3.

Together, the results of mass spectrum analysis, pharmacological activity measurements (filipin staining, Amplex Red cholesterol determination, and LysoTracker dye staining), and proteomic profiling analysis demonstrate that MβCD-3 is the most efficacious preparation among the three tested.

**DISCUSSION**

Cyclodextrin is a mixture of different isoforms with differing amounts of substitute groups. In contrast to traditional small molecule drugs, it is impossible to obtain a pure molecule of cyclodextrin. Therefore, the distinct batches of cyclodextrins have different numbers of substitute groups and variations in the average molecular weights. We examined the mass spectrum profiles of three MβCD preparations. We empirically found that they have varying average molecular weights and fluctuating amounts of substitute groups, even though two of them with different lot numbers were obtained from the same vendor with the same catalog number. These three MβCD preparations also exhibited altered pharmacological activity with varied efficacy on reduction of lysosomal cholesterol accumulation. These differences continued on through the ability of the various MβCDs to ameliorate dysregulated protein expression in NPC1 cells. These findings indicate the necessity of employing an analytical method to profile different cyclodextrin preparations to select the qualified one for therapeutic applications.

Cyclodextrin’s activity on reduction of lysosomal cholesterol accumulation has been extensively examined in NPC1 cells and animal models.24–27 In addition, HPβCD is currently in clinical trials for the treatment of NPC1 disease.28,29 The
mechanism of cyclodextrins on the reduction of lysosomal cholesterol accumulation and decrease of enlarged lysosomes has been linked to enhancement of lysosomal exocytosis and efflux of cholesterol out of cells.24,34 The effect of cyclodextrins on autophagy was also reported; this may contribute to its activity on the reduction of lysosomal lipid accumulation in NPC1 cells.46 Therefore, cyclodextrins may also have the potential for treatment of other lysosomal storage diseases.

Variable potencies and activities of cyclodextrins have been encountered and reported that may be related to different cells, different assays, and different sources of cyclodextrins. It has been found that the effects of cyclodextrins on autophagosome formation varied on different days after the treatment with cyclodextrins.47 The autophagosome level increased in the first 2 days after the treatment in NPC1 patient cells and then reduced to a similar level as WT cells. Different cells and tissues as well as different assays may also cause variable responses that have been reported elsewhere.4,30–34,47

In this study, we report the variable pharmacological effects of cyclodextrin due to the manufacture sources and batches of cyclodextrins. To avoid the discrepancy in measurements of cyclodextrin’s potency and efficacy in both in vitro and in vivo experiments due to the chemical components in cyclodextrins, it is important to set up a standard analytical method for selection of high-quality cyclodextrin for pharmacological experiments and clinical trials. We obtained three preparations of MβCDs from two commercial vendors and tested them in a series of analytical and pharmacological experiments. The mass spectrum analysis showed that different batches of MβCDs have different molecular weights with a unique combination of
substitution groups. The average methyl substitution number and the average molecular weights of MβCD-1 are the lowest at 10.26 and 1278.2, respectively; the values for MβCD-3 are the highest at 14.36 and 1339.6 (average molecular weight number), while the values for MβCD-2 are in the middle with values of 13.19 and 1314.0. Obviously, the highly methylated β-cyclodextrin is more lipophilic than less methylated β-cyclodextrins and is believed to interact well with free cholesterol.

In addition to the pharmacological experiment, we determined the MβCD effect on protein expression in the patient cells. We have selected and focused on a subset of 19 proteins that are dysregulated in NPC1 patient fibroblasts compared with the WT control cells. These 19 cellular proteins involve in lysosome formation, vesicle expansion, and completion during autophagy, SNARE interactions in vesicular transport, and steroid biosynthesis. Similar to the observed pharmacological activities, different batches of MβCDs showed varied effects.
Differential Efficacy of Various MβCDs on NPC1 Patient Fibroblasts

on recovery degree of expression of these 19 dysregulated proteins. MβCD-3 exhibited much better recovery of these dysregulated proteins in NPC1 fibroblasts compared with MβCD-1 and -2, consistent with the observation of the highest reduction of unesterified cholesterol level by MβCD-3.

In conclusion, we have developed a set of experimental methods to characterize different preparations of MβCD for selection of an appropriate one for use as a therapeutic agent. The mass spectrum analysis can reveal profiles of average molecular weight and side-chain methylation levels in different MβCD preparations. The pharmacological experiments include filipin cholesterol staining, Amplex Red cholesterol determination, and LysoTracker dye staining for enlarged lysosomes. The proteomic protein expression analysis examines the effect of different cyclodextrin preparations on the signature of dysregulated protein expression levels in NPC1 cells. The combination of these techniques provides more details of the characteristic properties of a specific preparation of cyclodextrin. This new approach has potential to be used as a quality control method for selection of efficacious cyclodextrin to be used as a therapeutic agent for treatment of NPC and other lysosomal storage diseases.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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**Abbreviations Used**

- **DHCR24**: 24-dehydrocholesterol reductase
- **DPBS**: Dulbecco’s phosphate-buffered saline
- **DTT**: dithiothreitol
- **EthD-1**: ethidium homodimer
- **GABARAP**: GABA (A) receptor-associated protein
- **GABARAPL1**: GABA (A) receptor-associated protein-like 1
- **GABARAPL2**: GABA (A) receptor-associated protein-like 2
- **HCD**: higher energy collision-induced dissociation
- **HDAC**: histone deacetylase
- **HPBCD**: 2-hydroxypropyl-beta-cyclodextrin
- **LAPTM4A**: lysosomal protein transmembrane 4 alpha
- **LC-MS/MS**: liquid chromatography–tandem mass spectrometry
- **MS**: mass spectrometry
- **MJCD**: methyl-beta-cyclodextrin
- **NPC1**: Niemann-Pick disease type C1
- **NSAF**: normalized spectral abundance factor
- **NSDHL**: NAD (P)-dependent steroid dehydrogenase-like
- **PFA**: paraformaldehyde
- **USE1**: unconventional SNARE in the ER 1
- **VAMP7**: vesicle-associated membrane protein 7
- **WT**: wild-type