Research Article

Hearing Loss and Otopathology Following Systemic and Intracerebroventricular Delivery of 2-Hydroxypropyl-Beta-Cyclodextrin

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ABSTRACT

Cyclodextrins are simple yet powerful molecules widely used in medicinal formulations and industry for their ability to stabilize and solubilize guest compounds. However, recent evidence shows that 2-hydroxypropyl- β -cyclodextrin (HP β CD) causes severe hearing loss in mice, selectively killing outer hair cells (OHC) within 1 week of subcutaneous drug treatment. In the current study, the impact of HP β CD on auditory physiology and pathology was explored further as a function of time and route of administration. When administered subcutaneously or directly into cerebrospinal fluid, single injections of HP β CD caused up to 60 dB threshold shifts and widespread OHC loss in a dose-dependent manner. Combined dosing caused no greater deficit, suggesting a common mode of action. After drug treatment, OHC loss progressed over time, beginning in the base and extending toward the apex, creating a sharp transition between normal and damaged regions of the cochlea. Administration into cerebrospinal fluid caused rapid ototoxicity when compared to subcutaneous delivery. Despite the devastating effect on the cochlea, HP β CD was relatively safe to other peripheral and central

organ systems; specifically, it had no notable nephrotoxicity in contrast to other ototoxic compounds like aminoglycosides and platinum-based drugs. As cyclodextrins find expanding medicinal applications, caution should be exercised as these drugs possess a unique, poorly understood, ototoxic mechanism.

Keywords: cochlea, hearing loss, ototoxicity, cyclodextrin, drug delivery

INTRODUCTION

Environmental factors such as noise and aging have been implicated in acquired hearing loss, but only a small group of ototoxic medicinal compounds have been well characterized. Drugs with a high risk of severe, permanent, hearing loss primarily include aminoglycoside antibiotics and platinum-containing anti-cancer agents. However, a recent study found that up to 1 % of FDA approved drugs have unknown ototoxic potential (Chiu et al. 2008). Cyclodextrins have been implicated as a new class of ototoxin that could pose a significant risk to hearing (Crumling et al. 2012; Ward et al. 2010).

Cyclodextrins are cyclic oligosaccharides, which are naturally derived from starch. Their chemical structure includes a central hydrophobic cavity, making them powerful solubilizers (Loftsson et al. 2005).

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Derivative forms like 2-hydroxypropyl- β -cyclodextrin (HP β CD) are generally considered benign (Gould and Scott 2005). Consequently, modified cyclodextrins are used in hundreds of pharmaceuticals and industrial products. In medicine, HP β CD enhances drug solubility, stability, and bioavailability (Loftsson et al. 2005). Cyclodextrins are currently used as excipients in over 30 pharmaceuticals (Loftsson et al. 2005), and their medical use is expanding rapidly. Since 1999, a multitude of patents have been granted utilizing cyclodextrins to enhance therapeutic drug delivery to the ear (for example, Gao 2005; Lichter et al. 2014; Magal 1999). In addition, the ability of these drugs to solubilize lipids has made them attractive for the treatment of atherosclerosis, Alzheimer's disease, infectious disease, and lipid storage disorders (Dass and Jessup 2000; Graham et al. 2003; Moriya et al. 1993; Ottinger et al. 2014; Yao et al. 2012). In light of their potential ototoxicity, the use of cyclodextrins in medicine brings new risks to hearing.

The treatment of lipid storage disorders such as Niemann-Pick Disease Type C (NPC) is one of the most promising applications of cyclodextrins. Recent excitement about the use of HP β CD to treat NPC has been tempered by evidence of ototoxicity in mice and cats (Crumling et al. 2012; Ward et al. 2010). NPC is a progressive neurological disease that typically leads to death by the second decade of life. Disease-causing mutations result in cholesterol storage in endolysosomal compartments, impairing lipid homeostasis and protein trafficking (Vanier and Millat 2003). Treatment with HP β CD normalizes cholesterol homeostasis, limits disease progression, and prolongs life in mouse models of NPC (Davidson et al. 2009; Peake and Vance 2012; Ramirez et al. 2010). Beta-cyclodextrins primarily act on membrane cholesterol (Kiss et al. 2010; Zidovetzki and Levitan 2007). In the NPC model, HP β CD appears to shuttle cholesterol from lysosomal to cytosolic compartments (Taylor et al. 2012). However, alterations in cell membrane and intracellular lipids could have important consequences on cell physiology. Cyclodextrins can mediate changes in ion channels, tight junctions, cellular transport, and cell signaling with potentially detrimental impact on cell function and viability (Ahsan et al. 2003; Casas et al. 2010; Francis et al. 1999; Kiss et al. 2010; Lynch et al. 2007; Purcell et al. 2011). While cyclodextrins have been studied in multiple organ systems, little is known regarding their interaction with the cochlea.

To better understand the impact of HP β CD ototoxicity, wild-type mice were treated under conditions reflective of current therapeutic doses used in human trials (ClinicalTrials.gov Number NCT01747135; Hastings 2009, 2010). Both peripheral

and central routes of administration proved ototoxic. Therapeutic doses, while relatively safe when given independently, had a compounding effect when administered concurrently. Deficits were attributable to the selective loss of outer hair cells (OHC). No other pathological findings were identified within the cochlea or in other major organ systems. HP β CD ototoxicity may be an acceptable compromise in an otherwise fatal condition such as NPC, but it may limit the broader applicability of this drug for treating other medical conditions. Careful audiological monitoring is highly recommended when using this compound.

METHODS

Animals and Treatments

Procedures involving the use of animals were approved by the University Committee on Use and Care of Animals at the University of Michigan. FVB/NJ mice (The Jackson Laboratory, stock # 001800) were used because of their relatively good hearing with no particular sensitivity to age-related hearing loss (Crumling et al. 2012; Zheng et al. 1999). Mice were 4–6 weeks of age at time of injection.

HP β CD (Sigma-Aldrich, H107) was prepared in 0.9 % NaCl and filter sterilized. Injections were administered either subcutaneously (SQ) at the nape of the neck or via stereotactic intracerebroventricular (ICV) delivery. SQ injections were delivered at up to 10,000 mg/kg body weight (b.w.), with controls given a comparable volume of sterile 0.9 % NaCl. The majority of animals were tested at either 3000 mg/kg (SQ Low) or 6000 mg/kg (SQ high). When normalized according to differences in body surface area between species (Reagan-Shaw et al. 2008), SQ low and SQ high correspond to 240 and 480 mg/kg, respectively, in humans. These concentrations were chosen based on human equivalent dose ranges initially scheduled in Compassionate Use trials for two NPC patients (Hastings 2009) but are well below escalated doses eventually reached in these and additional NPC patients (maximally 2900 mg/kg) (Hastings 2010; Matsuo et al. 2013).

Similarly, ICV injection doses were chosen based on therapeutic trials in humans (Hastings 2010) and animal studies (Ward et al. 2010). Escalating dose trials in humans had a goal of delivering 875 mg HP β CD by intrathecal injection, corresponding to approximate cerebrospinal fluid concentrations of 5 mM. In the present study, ICV injections were administered at 500 mg/kg (ICV low) and 3000 mg/kg (ICV high) by brain weight (br.w.), with control animals receiving an equivalent volume of sterile 0.9 % NaCl. Mouse brain weight was estimated

based on body weight for this strain (Rosen et al. 2000), and assuming a standard cerebrospinal fluid (CSF) volume of approximately 40 μ L (Segal 1993), the ICV low and high concentrations correspond to 5 and 30 mM, respectively. The volume of HP β CD injected into the ventricles was less than 4 μ L. ICV injection was performed using standard methods (Glascocock et al. 2011; Tsuneki et al. 2008). In brief, animals were anesthetized with 2 % isoflurane then secured in a stereotactic frame. The bregma was identified, and a craniotomy performed 1 mm lateral and 0.5 mm posterior to the suture. A 30 gauge needle on a 10- μ L gas-tight syringe was advanced to a depth of 2.5 mm from the brain surface to access the lateral ventricle. HP β CD was injected at 1 μ L/min, and the needle was left in place for 5 min to allow for distribution of the drug. To validate our approach, mice were injected with methylene blue, and the brains explanted and sectioned at various time points (data not shown), confirming distribution of the dye throughout the ventricles within minutes of injection.

Audiometry

Unless otherwise indicated, auditory thresholds were determined in control and treated mice 1 week after injection. Auditory brainstem responses (ABR) were conducted, as previously described (Crumling et al. 2012). Mice were initially anesthetized with ketamine (65 mg/kg, IP), xylazine (3.5 mg/kg, IP), and acepromazine (2 mg/kg, IP), and additional half doses of ketamine were given as needed to maintain depth of anesthesia. Mice were maintained on a heating pad during recordings in an electrically and acoustically shielded booth. Needle electrodes were placed ventral to each ear and in the vertex. The sound stimulus, consisting of 15 ms tone bursts, was then delivered into the ear canal via an encased, shielded Beyer earphone attached to a 13-mm tube. Response waveforms were recorded on a Tucker-Davis System 3 system. Both systems were calibrated in a closed volume approximating that of a mouse ear canal using a reference condenser microphone (Brüel & Kjær 1/8th inch, Type 4138) and a lock-in amplifier (Stanford Research Systems SR830) over a broad frequency range spanning the reported results. Responses were recorded at 4, 16, and 32 kHz which corresponded to apical, middle, and basal turns of the cochlea, respectively. Up to 1024 responses were averaged for each stimulus level. Each ABR trial began with a tone burst at 80 dB sound pressure level (SPL), but if no response was observed, the intensity was raised to 110 dB SPL. To identify a threshold response, sound intensity was decreased in 10 dB steps from either of these initial levels until approaching threshold at which point 5 dB steps were utilized.

Once no response was seen, the threshold was taken as a value between the lowest level leading to a reliable response and level of no response.

Whole-mounts of Organ of Corti and Cytocochleograms

Mice were euthanized under anesthesia. Temporal bones were excised, perfused, and fixed in 4 % paraformaldehyde overnight at 4 °C and decalcified in 5 % buffered EDTA (*w/v*). Apical, middle, and basal preparations of the organ of Corti were isolated and treated with 0.3 % Triton X-100 in phosphate buffered saline (PBS). After permeabilization, the preparations were washed with PBS three times and stained with Alexa 488-conjugated phalloidin (Invitrogen; 1:100) and imaged on an epifluorescence microscope. Phalloidin-labelled hair bundles were used to identify individual inner hair cells (IHCs) and OHCs. Successive 0.19 mm fields were counted using a calibrated reticule, and the percentage of missing hair cells determined using a normative database (KHRI Cytocochleogram, v3.0.6). Mapping of characteristic frequency to distance along the length of the cochlea was based on published maps for CBA/J mice (Müller et al. 2005).

Necropsy and Histopathology

At necropsy, tissues including skin (site of injection), brain, liver, spleen, kidney, and lung from each experimental group were collected for histopathologic analysis. Tissues were placed in 10 % neutral buffered formalin for fixation. Cross-sections of brain were obtained at the site of injection identified using tissue dye applied through the original craniotomy as a landmark. The dorsal calvarium was removed, and the brain was fixed in situ in the skull. Following fixation, all tissues were embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin. Tissue lesions were graded based on severity using a four-point severity scoring system, where 0=no lesion, 1=minimal sensitivity, 2=mild, 3=moderate, and 4=marked severity. In the brain, minimal severity lesions (grade 1) included those that did not extend beyond the superficial layers of the cortex at the injection site. Mild (grade 2) lesions were considered those that extended deeper into the cortex, but did not extend beyond the outer cortex to the hippocampus. Moderate (grade 3) lesions involved a larger zone of parenchyma within the outer cortex accompanied by degeneration of neurons within the underlying hippocampus. Marked lesions (grade 4) would involve regionally extensive necrosis of the cortical and hippocampal parenchyma. In the skin, minimal (grade 1) lesions were characterized as small, focal

inflammatory infiltrates and/or focal degeneration of few myofibers within the muscular layers of the skin. Mild (grade 2) lesions involved increased numbers of inflammatory cells and myodegeneration, and moderate (grade 3) lesions represented regionally extensive inflammation involving multiple layers of the skin accompanied by focal myodegeneration and small amounts of edema and granulation tissue. Marked lesions (grade 4) in the skin would be considered if there were extensive necrosis of the skeletal muscle layer or deeper subcutis, full-thickness inflammatory infiltration in a regionally extensive manner, or evidence of necrosis and ulceration of the dermis/epidermis.

Temporal bones were excised, decalcified, stained with paragon, and dehydrated in a series of ethanol (ETOH) solutions from 35 to 100 % ETOH. The cochleae were then infiltrated with a 1:1 solution of ETOH and JB-4 Glycol Methacrylate (JB-4 Plus) (Electron Microscopy Sciences) overnight. The sample was embedded in JB-4 Plus for 24 h and finally transferred to a 4 °C cooler to polymerize for 2 h. The embedded tissue was sectioned at 3 μ m thickness and mounted on glass slides. Twelve mid-modiolar sections were collected from each preparation. Digital images were acquired from 6, non-successive sections and analyzed with MetaMorph Image Analysis Software under bright field optics. Spiral ganglion cell (SGC) number and density were determined as described elsewhere (Miller et al. 2007). Briefly, the total area of the basal, mid, and apical Rosenthal's canals was measured and SGC number manually counted. SGC density was calculated as the number of SGCs divided by the canal area. The data from all six sections were averaged for each preparation. Tissue sections were also evaluated based on the four-point severity score for lesions in the stria vascularis, lateral wall, and Rosenthal's canal. In all areas, minimal lesions (grade 1) included restricted regions of edema and vacuolization, whereas mild to moderate lesions (grades 2 to 3) included expanding levels of edema and evidence of necrosis. Marked severity (grade 4) was evidenced by widespread cell loss.

Statistics

Statistically significant differences in mean ABR thresholds for each frequency were tested across groups using one-way ANOVA with post hoc comparisons between groups using a Tukey test. Differences in mean SGC density were also tested by one-way ANOVA across treatment groups. Exact *P* values are provided when possible. Statistical tests were implemented in SYSTAT 13. Unless otherwise indicated,

averaged data are reported as means $\pm$ one standard error of the mean.

RESULTS

HP β CD Hearing Loss

In a previous study, single SQ doses of HP β CD were capable of inducing large, dose-dependent shifts in ABR threshold across a wide range of test frequencies (Crumling et al. 2012). The current study replicated the SQ dose effect and extended the observation of ototoxicity to centrally delivered HP β CD. Dose-dependent shifts in ABR thresholds were identified in both SQ- and ICV-treated animals. Subjects receiving the SQ high dose (6000 mg/kg b.w.) exhibited elevated thresholds across all test frequencies (Fig. 1A) ($P<0.001$). Mean threshold shifts compared to control groups were 35 to 45 dB, consistent with high dose shifts reported previously (Crumling et al. 2012). Likewise, ICV high (3000 mg/kg br.w.) increased thresholds at all frequencies ($P<0.001$) with mean threshold shifts of 50 to 60 dB, similar to SQ high HP β CD (Fig. 1A). There was no statistical difference between threshold shifts for SQ high or ICV high at any frequency (4 kHz $P=0.49$; 16 kHz $P=0.26$; 32 kHz $P=0.29$), indicating that a common mechanism may be involved regardless of route of administration. To evaluate this possibility further, SQ high and ICV high doses were concurrently administered to determine if combination dosing caused even greater auditory deficits. Again, ABR thresholds were elevated over controls, but the magnitude was similar to individually administered high doses (vs. SQ high: 4 kHz $P=0.95$; 16 kHz $P=0.95$; 32 kHz $P=0.86$; vs. ICV high: 4 kHz $P=1.00$; 16 kHz $P=0.99$; 32 kHz $P=1.00$) (Fig. 1A). The results support the notion that a common mechanism and a common target are involved in both routes of administration.

SQ low (3000 mg/kg b.w.) and ICV low (500 mg/kg br.w.) doses were determined based on previous experiments in mice (Crumling et al. 2012) and cats (Ward et al. 2010) and on treatment schedules for the phase I trials (ClinicalTrials.gov Number NCT01747135). Neither regimen alone caused substantial shifts in ABR thresholds compared to controls; only ICV low resulted in a significant shift in threshold at any frequency (32 kHz $P<0.001$, about 30 dB above control mean) (Fig. 1B). However, when combined, SQ low plus ICV low caused a dramatic ABR threshold shift at all frequencies ($P<0.001$) (Fig. 1B). Moreover, the mean thresholds for combined SQ low and ICV low were similar to the high doses delivered together (vs combined SQ high and ICV high: 4 kHz $P=0.97$; 16 kHz $P=1.00$; 32 kHz $P=1.00$). This synergy at low doses again suggests both a common mechanism and

FIG. 1. ABR thresholds were elevated 1 week after HPβCD treatment by SQ or ICV injection. Mean thresholds of control, SQ, ICV, and combined cohorts for high dose (A) and low dose (B) HPβCD are shown for three test frequencies. Saline vehicle controls for SQ and ICV injections were combined for a single control group since there was no statistical difference between these individual control groups (Student's two-tailed *t* test for each frequency: 4 kHz $P=0.27$; 16 kHz $P=0.19$; 32 kHz $P=0.15$). Elevated thresholds were observed for all high dose groups compared to controls, whereas only concurrent administration of SQ low and ICV low resulted in similarly elevated thresholds across all test frequencies. Statistically significant differences in mean ABR thresholds for each frequency were tested across groups using one-way ANOVA (4 kHz: $F=16.85$,

$df=53$, $P<0.001$; 16 kHz: $F=26.68$, $df=53$, $P<0.001$; 32 kHz: $F=30.55$, $df=53$, $P<0.001$). Post hoc pairwise comparisons between groups was performed using a Tukey test with significant differences from controls indicated by an asterisk (* $P<0.001$). The number of animals per group: control (15), SQ low (6), ICV low (9), SQ+ICV low (6), SQ high (12), ICV high (8), SQ+ICV high (4). Histograms of control ABR thresholds (gray-shaded region) and all HPβCD treatment groups (filled black symbols) are plotted as threshold shift from control means for all test frequencies (C). The HPβCD data was best fit (Simplex algorithm) with a two-component Gaussian function, revealing one component centered with control data and the other component centered at ~57 dB.

a common target of SQ and ICV administration and underscores the potential ototoxicity of what otherwise appears to be a benign drug at low doses.

Previously, HP β CD effects on ABR thresholds appeared to group into two distributions (Crumling et al. 2012), namely animals with normal thresholds (unaffected) and those with elevated thresholds (affected). A similar result was found in the current study. A histogram of all data from HP β CD treated animals, normalized as threshold shift from control means, revealed two distinct response populations, one that overlaps with control data and one that is tightly grouped around an average shift of 57 dB (Fig. 1C). Animals were segregated into affected and unaffected groups using the 95 % confidence interval for control data as a criterion; specifically, those animals with thresholds at all three test frequencies outside the control group 95 % confidence interval were identified as “affected.” SQ high affected 83 % of animals, causing the characteristic 40–60 dB threshold shifts at all test frequencies in those animals. Interestingly, ICV injections showed variable sensitivity as well, but only at the low dose end. ICV low affected 22 % of the animals with 40–60 dB shifts, whereas ICV high affected 100 % of the animals with large threshold shifts at all test frequencies. It remains unclear whether the variable impact of HP β CD in some conditions is related to a steep dose–response or variability in SQ injection depth as well as differences in local tissue composition that could impact drug kinetics and therefore ototoxicity. The observation of bimodal distributions in treated cohorts from this and our previous study suggests a biological origin and raises the possibility of a quantal, all-or-none response threshold.

HP β CD Ototoxicity

The widespread loss of OHCs has been implicated in HP β CD ototoxicity (Crumling et al. 2012). To determine whether OHCs remain the primary target of both ICV and SQ administration, whole mounts of the organ of Corti were examined 1 week after injection. Sporadic OHC loss was observed in mid-apical preparations from control animals (Fig. 2A). Rings of phalloidin-labelled actin are seen in the gaps of missing hair cells, marking the scars formed by surrounding supporting cells that infiltrate into these spaces. Similar scar patterns are seen throughout the mid-apical segments of SQ high and ICV high preparations (Fig. 2B, C), revealing 100 % loss of OHCs in these regions and indicating that the surrounding supporting cells remain intact. Low doses resulted in a normal complement of hair cells, except when administered concurrently resulting in widespread OHC damage (Fig. 2D). Cytochrome

were used to quantify the degree of hair cell loss. In each treatment group, three to six animals were chosen at random for whole-mount preparation and hair cell quantification. Inner hair cells were unaffected by the drug treatment or vehicle control, regardless of dose level or route of administration (Fig. 2E, G, and I). In contrast, conditions that led to elevated ABR thresholds were accompanied by OHC loss (Fig. 2F, H, and J). Data from ICV low included variable OHC loss at the cochlear base (Fig. 2F), mirroring the variable but significant elevation in ABR thresholds for 32 kHz tone bursts in this group. OHC loss was widespread in the SQ and ICV high treatment groups (Fig. 2H) and in the SQ+ICV combined groups (Fig. 2J). The incomplete penetrance of hearing loss from SQ dosing was reflected in the intermediate level of cell loss, averaged across affected and unaffected animals. The degree of OHC loss on an individual animal basis was tightly correlated with corresponding threshold shifts. The composite OHC loss across the cochlea was plotted against the threshold shift for a 16-kHz tone burst relative to control data (Fig. 3). The bimodal response to HP β CD is apparent, with only one animal (ICV Low) exhibiting an intermediate threshold shift and intermediate level of OHC loss.

To begin elucidating the kinetics of HP β CD ototoxicity, we evaluated cytochrome at various time intervals after SQ high and ICV high doses. OHC loss from SQ injection was apparent 1 day post-injection (Fig. 4A). Over the next several days, the mean percentage of OHCs lost from SQ high steadily increased (Fig. 4A, C). Interestingly, the time-dependent increase in mean OHC loss resulted primarily from an increase in the proportion of ears with near complete loss of OHCs rather than a gradual loss of cells over time. After 0, 1, 3, and 7 days post-injection, the percentage of animals exhibiting >85 % OHC loss was 0, 17, 33, and 83 %, respectively. There was considerable interanimal variance in the onset of OHC loss after SQ drug injection and thus variance in the kinetics of HP β CD accumulation. Further, we rarely observed intermediate levels of damage, indicating a rapid progression from onset of injury to widespread OHC loss. As a result, it was difficult to ascertain the progression of injury along the cochlea from these snapshots every few days. Insight in this regard was more readily gleaned from the ICV high preparations, where a base-to-apex progression over time was evident (Fig. 4B). In this case, the transition between normal OHC density and damage was seen progressing from 2.5 mm from the apex after 2.5 h, 1.5 mm after 5 h, and 1 mm after 18 h. Although both administration routes targeted the same population of cells and ultimately to the same extent, the pharmacokinetics and progression of

FIG. 2. Hair cell loss from HP β CD is primarily restricted to OHCs. Mid-apical segments of the organ of Corti are shown for control (A), SQ high (B), ICV high (C), and combined SQ+ICV low (D) preparations. Percent missing IHCs (E, G, I) and OHCs (F, H, J) are plotted for each treatment condition from animals selected at random, without consideration of ABR response. Control data are

re-plotted in each panel for comparison. Legend in E applies to F, G to H, and I to J. The number of animals per group is shown in parentheses alongside each legend. A map of cochlear location to characteristic frequency is illustrated in (E).

injury were different and could have important implications for monitoring clinical use of the drug.

HP β CD Pathology

Plastic sections of the cochlea and other organs were evaluated for drug-related pathology beyond OHC loss. Significant pathology findings were subjectively scored in a blinded manner using a 4-point scale that progressed from no apparent injury, to cell swelling, and to widespread cell loss. The data are summarized in Table 1 indicating the average score for six independent preparations and the percentage of preparations with a score of 1 or greater in each condition. Overall, only mild effects were seen. Few of these effects were clearly drug related or potentially deleterious to the animal. In the ear, minimal damage

was noted in the stria vascularis and lateral wall, limited primarily to edema and vacuolization in fibrocytes and marginal/intermediate cells (Fig. 5A–D). Less than half of the preparations receiving SQ high, ICV high, or both exhibited this damage; those exhibiting the most severe damage are shown in (Fig. 5B–D). No statistically significant difference in SGC density was found despite the difference in HP β CD administration route (Fig. 5E–H; $F=2.7$, $df=14$, $P=0.06$). There was no correlation between ABR thresholds and damage to the other cochlear structures.

Outside of the ear, treatment-related lesions were identified in the skin from SQ injections. All other tissues showed no clear evidence of drug-related pathology (Table 1). Locally extensive parenchymal necrosis was observed in the brain at the site of ICV

FIG. 3. The extent of OHC loss was highly correlated with ABR threshold. The average % hair cell loss is plotted against the mean threshold shift at 16 kHz (relative to control data) for individual animals contributing to data in Fig. 2. Control data (filled symbols) are shown alongside HP β CD treatment groups (open symbols) for percent missing IHCs (circles) and OHCs (triangles). There is a normal complement of IHCs regardless of the impact of HP β CD on hearing sensitivity. In contrast, elevated ABR thresholds were uniformly associated with greater OHC loss ($N=39$).

injection in both control (Fig. 6A) and HP β CD-injected mice (Fig. 6B). This lesion was characterized by loss of architecture within the white matter of the outer and inner cortex, superficial fragmentation of the neuropil, infiltration by a few microglial cells, and variable neuronal necrosis within the hippocampus evidenced by shrunken, angular, and hypereosinophilic neurons, with variable focal hemorrhage. Higher dose HP β CD skin injection sites showed variable mild to moderate dermatitis, cellulitis, and myositis, whereas control animals injected with saline had minimal dermatitis (Fig. 6C, D). In addition, animals receiving SQ high HP β CD had large numbers of eosinophils present within the inflammatory lesions compared to a predominantly mononuclear inflammatory response in SQ-injected control animals. Focal and minimal hepatocellular necrosis was observed in sections of liver from two control animals and two ICV-treated animals. This lesion is a background lesion occasionally observed in mice and occurred in both treated and control mice. Similarly, diffuse lymphoid hyperplasia was noted in the spleen in nearly half of control and treated animals. Since these lesions are common normal background variation, they were not considered to be related to treatment. In the lung, ICV-treated animals often presented with acute hemorrhage characterized by congestion and filling of alveolar spaces by erythrocytes. Since there was no evidence of vascular damage, inflammation, pulmonary toxicity, or other lung disease histologically, the observed acute pulmonary hemorrhage was considered to be iatrogenic due to blood collection

FIG. 4. OHC loss progressed over days for SQ high (A) and hours for ICV high (B). The percent missing OHC were averaged from randomly selected animals in each condition and at each time point. SQ high data are shown for the whole cohort (open triangle) and a subset of affected animals (closed square). Hour time points for ICV high groups were collected over a time range, $\pm 20\%$ of the midpoint indicated in the legend. Only positive error of the mean is shown for each point for clarity. The number of animals per group is shown in parentheses alongside legends. The mean composite loss of OHCs throughout the full length of the organ of Corti is shown (C).

from the heart at the time of euthanasia prior to necropsy. Lesions of the kidney were not observed in any cohort (Fig. 6E, F).

DISCUSSION

Cyclodextrin use in medicine has gained momentum in recent years, particularly with the emergence of HP β CD as a promising treatment for NPC (Ottinger et al. 2014). Though once considered primarily an excipient and inactive pharmaceutical compound, a concerted bench-to-bedside effort in animal models of NPC opened the door to using uncomplexed HP β CD as a therapeutic agent (Pontikis et al. 2013;

TABLE 1
Severity and Incidence of Histopathologic Findings

Diagnosis	Control SQ	SQ	Control ICV	ICV	ICV+SQ
Cochlea (stria vascularis)	0 ^a	0	n.d.	0.2 (17 %) ^b	0
Cochlea (lateral wall), vacuolization	0	0.3 (33 %)	n.d.	0.5 (50 %)	0.3 (33 %)
Brain, necrosis	0	0	2.2 (100 %)	2.6 (100 %)	2.37 (100 %)
Skin, dermatitis	1.2 (100 %)	2.4 (100 %)	0	0	0.3 (33 %)
Liver, necrosis	0.3 (33 %)	0	0	0.4 (40 %)	0
Spleen, lymphoid hyperplasia	0.5 (33 %)	0.6 (40 %)	0.2 (17 %)	0.4 (20 %)	1.3 (67 %)
Lung, hemorrhage	0	0	0	0.6 (60 %)	1.3 (50 %)

n.d. not determined

^aAverage severity score based on a four point system (0=no significant findings; 1+=minimal; 2+=mild; 3+=moderate; 4+=marked)

^bPercentage of animal preparations receiving a non-zero score

Ward et al. 2010). Due to poor blood–brain barrier permeability (Pontikis et al. 2013), effective treatments for NPC and other lipid storage disorders will undoubtedly require both peripheral and central administration. However, in the current study, we showed that peripheral and central administration of HP β CD was ototoxic at therapeutic dose levels. Singular high-dose injections, regardless of route, caused ABR threshold shifts of 40 to 60 dB across a wide range of test frequencies. Concurrent injection by SQ and ICV routes, regardless of dose level, caused similar deficits. Threshold shifts were consistent with loss of the cochlear amplifier through the death or dysfunction of OHCs (Liberman et al. 2002; Ryan and Dallos 1975). Cytochrome c revealed widespread loss of OHCs from both ICV and SQ HP β CD, with near complete loss extending from the base to approximately a characteristic frequency of 8 kHz.

ABR thresholds at 4 kHz were elevated, although OHCs remained intact in apical, low-frequency regions. This apparent discrepancy could be explained by residual dysfunction in those remaining OHCs (Crumling et al. 2012) and/or loss of hair cells in slightly more basal regions that can contribute to suprathreshold responses at low frequencies (Taberner and Liberman 2005). Inner hair cells were well preserved in all cases. Likewise, normal complements of SGCs were found in all treatment groups. Drug-related effects were found in the lateral wall of some animals, but the extent of injury was not correlated with differences in ABR thresholds. Taken together, the results suggest that HP β CD is able to infiltrate the cochlea despite different routes of administration and cause OHC death through a common mechanism without impacting the remainder of the cochlea. Other sub-lethal pathophysiological

FIG. 5. Plastic sections of control and HP β CD treated animals revealed only minor degeneration outside of the organ of Corti. Basal turns are shown for control (A, E), SQ high (B, F), ICV high (C, G), and combined SQ and ICV high (D, H). A–D Drug effects were absent in stria vascularis, but drug-dependent vacuolization of the lateral wall was apparent in some HP β CD treated animals, particu-

larly in the region of type I fibrocytes. E–H SGC shape and density was unaffected by drug treatment. Scale bar in (H)=50 μ m and applies to all panels. Images are representative of the most severe lesions in 6 independent preparations per condition.

FIG. 6. ICV injections in vehicle control mice (**A**) and HPβCD-treated mice (**B**) had similar lesions in brain related to injection trauma, including degeneration and necrosis of the neuropil (*arrow-heads*). SQ vehicle administration (**C**) elicited few inflammatory cells within the skin, whereas SQ administration of HPβCD (**D**) elicited a regional dermatitis, cellulitis, and myositis. There were no histologic

differences between kidneys in mice treated with vehicle (**E**) compared to mice treated with HPβCD (**F**). Images are representative of the most severe lesions in 6 independent preparations per condition.

ical effects may be present since ABR thresholds were highly elevated or even undetected in some animals. The data raises caution about the clinical use of HPβCD, particularly if delivered directly into CSF and if used as part of a chronic treatment regimen.

The kinetics of HPβCD ototoxicity was rapid, beginning within a day of SQ injection and only hours after ICV injection. Low doses acted synergistically to cause ototoxicity, despite suspected differences in the kinetics of SQ and ICV administration with regard to entry and elimination of the drug from the cochlea. The results indicate that either drug clearance was slow or that drug effects on OHCs accumulated and were long-lasting. The speed of OHC loss and selective nature of the injury suggests direct action on these cells and, therefore, penetration into the cochlear duct. SQ injections likely transmitted HPβCD

to inner ear fluid spaces via cochlear vasculature, whereas entry following ICV injections could also arise from multiple, direct communication routes between CSF and the inner ear. These central routes include a large, patent cochlear aqueduct in rodents and perineural/perivascular spaces subarachnoid spaces to the cochlear modiolus via the internal auditory meatus (Nakashima et al. 2012). These paths would allow accumulation of HPβCD into the fluid spaces surrounding the basolateral OHC membrane. The relative contribution of these routes from ICV injection in mice is unclear, which makes it difficult to speculate how ICV HPβCD might impact other species due to variations in the patency of the cochlear aqueduct. In humans, for example, the cochlear aqueduct is small and filled with dense connective tissue (Toriya et al. 1994). Further studies are needed

to define the pharmacodynamics and penetration into the ear in order to predict the impact of cyclodextrin in the human population.

The mechanism of HP β CD ototoxicity remains unknown. HP β CD is thought to work primarily through cholesterol modulation (Ramirez et al. 2010), but it has been shown to complex with other phospholipids and lipids within cells (Zidovetzki and Levitan 2007). Cyclodextrins have been studied in many cellular and organ systems and can affect cellular cholesterol content within hours (Taylor et al. 2012), consistent with the timing of injury from ICV injection. While it is tempting to assign the mechanism of HP β CD ototoxicity to a yet undiscovered interaction with OHC cholesterol, it is unclear why OHCs are selectively targeted since cholesterol is ubiquitous in mammalian cells. OHCs have an inhomogeneous distribution of free cholesterol with higher concentrations in the basal and apical membranes and lower levels in the lateral wall (Nguyen and Brownell 1998). These domains correspond to segregated membrane proteins, Prestin in the lateral wall (Ashmore 2008) and KCNQ4 channels at the base (Mustapha et al. 2009), suggesting a unique relationship between these cholesterol domains and membrane function. Changes in cholesterol content of OHCs affect OHC electromotility and Prestin function leading to changes in the cochlear amplifier (Brownell et al. 2011). Likewise, changes in cholesterol can inhibit KCNQ subtypes (Lee et al. 2010). Interestingly, Prestin and KCNQ4 mutants exhibit progressive OHC loss (Kharkovets et al. 2006; Liberman et al. 2002), indicating that dysfunction of these key proteins could contribute to cell death. Therefore, it is possible that the unique structure and function of OHCs make them especially sensitive to disruption in cholesterol homeostasis relative to other somatic cells. Additionally, cyclodextrins have been shown to disrupt tight junctions (Ahsan et al. 2003), decrease transepithelial resistance (Francis et al. 1999), increase calcium currents, and decrease rectifying K⁺ currents (Purcell et al. 2011). Consequently, HP β CD may act upon several targets that combine to enhance the sensitivity of OHCs over other cell types. For example, a disruption of tight junctions in the reticular lamina could lead to leakage of K⁺ into spaces of Nuel surrounding OHCs, depolarizing these cells, exacerbating calcium influx, and ultimately contributing to excitotoxicity.

Other well-characterized ototoxins include the platinum-based chemotherapeutic agents (e.g., cisplatin) and aminoglycoside antibiotics (e.g., gentamicin and neomycin). While these therapeutics have their own unique features, both primarily target

OHCs, generate reactive oxygen species (ROS) as key initiating factors in cell death, and exhibit a base to apex pattern of cell death and injury. Since it is known that the cochlea exhibits decreased sensitivity to oxidative stress from base to apex (Ohinata et al. 2003; Schacht et al. 2012; Usami et al. 1996) and the basal to apical pattern of OHC loss seen in our study parallels that of aminoglycosides and cisplatin, it is possible that HP β CD mediates its effects through ROS. There is evidence to suggest that β -cyclodextrin derivatives are capable of altering mitochondrial bioenergetics (Ziolkowski et al. 2010), and this could be through direct action on intracellular organelles (Rosenbaum et al. 2010). It is unclear whether HP β CD accumulates in OHCs, but it remains possible that HP β CD ototoxicity is mediated through a direct interaction with intracellular signaling and ROS production. While cyclodextrin ototoxicity shares many similarities to aminoglycosides and platinum-based compounds, the most striking difference is the lack of nephrotoxicity. Both cisplatin and aminoglycosides are nephrotoxic (Ali et al. 2011; dos Santos et al. 2012), yet HP β CD did not cause kidney damage.

HP β CD is ototoxic in mice and cats, but it remains to be shown if equivalent doses will be ototoxic in human trials. While standard formulas exist to convert animal doses to human doses, these are based on body surface area measurements, which may have no bearing on drug dynamics. In humans, differences in the anatomy of the cochlear aqueduct, size of the cochlea, volume of the cochlear fluids, and rates of longitudinal flow within the cochlea may affect the entry of drug into the cochlea, its clearance after ICV injection, and therefore the extent of damage compared to results in mouse (Salt 2002). Although both normal and NPC cats were susceptible to HP β CD ototoxicity, cellular cholesterol content and unique manifestations of NPC in the various human alleles could affect human sensitivity to the drug. Human sensitivity to HP β CD has been observed in an ongoing clinical trial for NPC patients, where ototoxicity is now recognized as a likely clinical outcome (King et al. 2015). However, it remains to be seen whether the extent of injury is as severe in NPC patients receiving therapeutic dose levels in chronic treatment paradigms compared to the single, high doses used here. Moreover, the potential impact on hearing must be balanced with the efficacy of HP β CD to treat NPC. Even so, cyclodextrins are candidate therapeutics for other less life-threatening diseases, where ototoxicity may be the limiting side effect. Further studies to elucidate the mechanism of this unique ototoxin are needed.

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