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The Histone Deacetylase Inhibitor Sodium Butyrate Protects Against Cisplatin-Induced Hearing Loss in Guinea Pigs

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Abstract

Objective: There is a need for otoprotective agents that can be administered systemically without compromising cancer treatment. Histone deacetylase inhibitors are anticancer agents that act by upregulating the expression of cell-cycle control genes. They are also neuroprotective, leading us to hypothesize that they might be otoprotective. The goal of this study was to determine if the antitumor agent sodium butyrate (a histone deacetylase inhibitor) protects against cisplatin ototoxicity when administered systemically.

Study Design: This was an animal study.

Methods: Cisplatin was administered to guinea pigs who received either 12 days of sodium butyrate (7 d before and 5 d after cisplatin) or equivalent volume saline injections. Hearing was tested with distortion product otoacoustic emission (DPOAE) analysis before the start of the study and 2 weeks after cisplatin treatment.

Results: Guinea pigs given a single intraperitoneal injection of 14 mg/kg cisplatin experience a mean hearing loss of 8 dB across the frequencies of 3.5, 5, 7, 10, 14, and 20 kHz. Intraperitoneal injection of 1.2 mg/kg sodium butyrate per day for 7 days before and 5 days after cisplatin almost completely eliminates this threshold shift ($P = .0011$).

Conclusions: The histone deacetylase inhibitor sodium butyrate gives almost complete protection in a single-dose model of cisplatin ototoxicity in guinea pigs. Because histone deacetylase inhibitors are anticancer agents with very few side effects, they may be candidates for clinical use during cisplatin chemotherapy.

Keywords

Cisplatin; HL; histone deacetylase inhibitors; sodium butyrate; otoprotection

INTRODUCTION

Cis-diaminedichloroplatinum (cisplatin, CDDP) and carboplatin are essential components in the treatment of many tumors. Toxicities include bone marrow suppression, nephrotoxicity, and hearing loss (HL). HL begins in the high frequencies but can progress to involve all

frequencies.¹ Cisplatin damages stereocilia, mitochondria, and nuclei of hair cells, ultimately leading to apoptosis, in a basal-to-apical and outer-to-inner progression.²

In the normal cochlea, reactive oxygen species (ROS) are a byproduct of normal metabolism; they are rendered harmless by antioxidant defense mechanisms. Trauma creates oxidative stress and can lead to ROS overwhelming the cellular antioxidant defenses.³ Because the ROS pathway is the common effector for many causes of HL, there is great interest in treatments that would prevent cell death as a result of ROS.

Several antioxidants can reduce or prevent ROS-induced damage and stop the progression to apoptosis.⁴⁻⁶ Antioxidant therapy might be clinically useful in preserving hearing, but there is evidence that antioxidants may interfere with the tumoricidal action of cisplatin.⁵

One promising alternative is local antioxidant therapy. Antioxidants delivered to the middle ear diffuse across the round window to the inner ear, where they can protect hearing without compromising cisplatin effectiveness in an animal tumor model.⁵ However, local delivery in humans would require additional invasive procedures (e.g., implantation of pumps and/or cannulae), and it may be difficult in uncooperative patients, particularly children.

Another option is to identify agents that are otoprotective but do not interfere with tumor kill. Histone deacetylase inhibitors (HDACis) may be such agents. HDACis and their complementary histone acetylases (HACs) regulate the acetylation of histones. Histone acetylation leads to looser coupling of DNA to the histones, which favors transcription. Histone deacetylation leads to tighter coupling of DNA and histones, which reduces transcription. HDACis, by favoring acetylation, favor transcription. In theory, HDACis should increase transcription of all genes, but in fact they specifically increase transcription of a very small subset (approximately 2%). Most prominently, they increase transcription of genes whose products control the cell cycle; HDACis inhibit uncontrolled cell division and have anticancer activity both in vitro and in vivo.^{7,8} Recent clinical trials have shown that members of the HDACi family are well tolerated and have antitumor activity in solid and hematologic tumors.⁸

HDACis are also neuroprotective in many models. They arrest Huntington's-like neurodegeneration and reduce the associated mortality in *Drosophila*.⁹ They are protective against oxidative stress in vitro and chemical insults in vivo.¹⁰ The molecular mechanisms of the neuroprotective effect appear to be mediated by acetylation of other cellular proteins (not histones). For example, acetylation of the transcription factor Sp1 in response to oxidative stress leads to transcription of protective factors. Sodium butyrate increases stress-induced Sp1 acetylation and may thus augment normal protective mechanisms.¹¹

Cochlear sensory cells share many features in common with neuronal cells. We therefore hypothesized that sodium butyrate would prevent cisplatin-induced damage and hence preserve hearing. The goals of this study were: 1) to develop a single-dose model of cisplatin-induced HL in the guinea pig and 2) to determine if the HDACi sodium butyrate would prevent or reduce this loss.

MATERIALS AND METHODS

Reagents

N-Butyric acid, sodium (sodium butyrate; Sigma-Aldrich Chemical Co., St. Louis, MO) was mixed aseptically as a 200 mg/mL solution with sterile saline before injections. Cis-platinum (II) diamine dichloride (cisplatin, CDDP) (Sigma-Aldrich Chemical Co.) was mixed fresh under aseptic conditions in sterile saline 24 hours before injection to a concentration of 0.25%

(weight/ volume). Sterile 0.9% saline (Abbott Laboratories, N. Chicago, IL) was injected in the control animals.

Animals

Eighty Hartley albino female guinea pigs (Charles River Laboratories, Wilmington, MA) with a starting weight of 250 to 300 g were used. Table I itemizes the numbers used in each experiment. Animals were housed in a single-species, temperature-controlled, 12-hour light/dark cycle facility. Water and food were provided ad libitum. Four animals were excluded for asymmetric hearing or thresholds >70 dB sound pressure level (SPL) on initial testing. Animals receiving cisplatin were inspected hourly for 8 hours postinjection and daily thereafter. These studies were approved by the Institutional Animal Care and Use Committees at Children's Hospital Boston, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary.

Anesthesia

Animals were given intraperitoneal injections (IP) of 30 mg/kg ketamine (Ketaset, Fort Dodge, IA) and 10 mg/kg xylazine (Xyla-Ject; Phoenix Scientific, St. Joseph, MO) before hearing tests. The anesthesia usually lasted approximately 2 hours, and no animals had side effects.

Distortion Product Otoacoustic Emission Testing

After induction of anesthesia, ear canals were examined with otoscope, excess wax and debris removed, and hair clipped. DPOAEs were measured using a custom acoustic assembly consisting of two electrostatic drivers (ED-1; Tucker Davis Technologies, Alachula, FL) as sound sources and a Knowles Electret microphone coupled to a probe tube to measure ear canal sound pressure. Two primary tones ($f_2:f_1 = 1.2$) were presented with f_2 level 10 dB < f_1 level. Stimuli were generated digitally, but attenuation was provided with analog attenuators. Ear canal sound pressure was filtered (high pass at 1000 Hz), amplified, and digitized by an A-D board. A fast Fourier transform was computed and sound pressures at f_1 , f_2 and $2f_1-f_2$ extracted after both waveform and spectral averaging of the ear canal sound pressure waveform. The noise floor, defined as the average of the six spectral points on either side of the $2f_1-f_2$ point, ranged between -20 and 5 dB SPL. At most sessions, each ear was tested twice at f_2 frequencies of 3.5, 5, 7, 10, 14, and 20 kHz. In a few cases, the animal awoke before the second tests could be completed. At each f_2 frequency, input sound pressure level was varied from 0 to 80 dB SPL in 5-dB steps.

Data Collection and Statistical Analysis

MATLAB programs were used to interpolate "thresholds" from the DPOAE amplitude versus level curves at each f_2 frequency. "Threshold" was defined as the input level of the primary tone f_2 required to produce a DPOAE at $2f_1-f_2$ that exceeded the noise floor by 5 dB. Threshold shifts for each ear were calculated by comparing pre- and postcisplatin results. Threshold shift was calculated for each ear separately. The average of these two shifts was used as one data point for statistical comparisons. Two-way analysis of variance (ANOVA) (MATLAB statistics toolbox) and Student *t* test (Excel) were used for statistical calculations.

Establishing Cisplatin Toxicity and Dose, and Sodium Butyrate Toxicity

A total of 35 animals were given a single dose of 8, 10, 12, 14, or 16 mg/kg cisplatin to establish a model with measurable HL and limited morbidity.

The dose of 1.2 g/kg sodium butyrate tested was chosen from previous work in a mouse model (Ryu and Rata, unpublished observations). To determine if sodium butyrate was ototoxic to

guinea pigs, five animals had a 13-day course of 1.2 g/kg IP sodium butyrate and pre- and posttreatment hearing tests.

Butyrate and Control Experimental Groups

A total of 36 animals with normal and symmetric hearing were matched for weight and assigned randomly to either the butyrate (n = 17) or the control group (n = 19, of which three died). Six animals were studied in each “batch,” and both control and butyrate animals were included in every “batch” to reduce possible variability as a result of colony health, time of year, and so on. Sodium butyrate or equivolume saline injections were given for 7 days before and 5 days after cisplatin. Cisplatin was given as a single injection of 14 mg/kg. Hearing was tested 2 weeks after the end of the injections. All injections were given at approximately 9 AM, and daily health checks were made. Figure 1 is a flow chart of the study design.

Stability of Hearing Loss at 8 Weeks

To ensure that a 2-week interval after cisplatin injection was adequate for hearing to stabilize, two control animals and two butyrate animals had hearing tests at 2 weeks posttreatment and again at 8 weeks posttreatment. These animals had no change in their hearing (data not shown).

RESULTS

Single-Dose Cisplatin Effects

Figure 2 shows the effects of a single injection of cisplatin at doses of 8 mg/kg (n = 5), 10 mg/kg (n = 5), 12 mg/kg (n = 10), 14 mg/kg (n = 10), and 16 mg/kg (n = 5). A measurable HL was defined as an average increase in threshold of at least 5 dB from the pretest value. Below 14 mg/kg, no more than 10% of animals had a measurable loss. Above 14 mg/kg, there was 40% mortality. At 14 mg/kg, nine of 10 animals had measurable HL and one of 10 animals died. Therefore, this dose was used for subsequent studies.

No Evidence of Butyrate Toxicity

Guinea pigs receiving a 13-day course of sodium butyrate without cisplatin (n = 5) had no change in their hearing and no observable behavioral toxicity (data not shown).

Toxicity in the Experimental Group

Animals receiving 14 mg/kg cisplatin and sodium butyrate protection had no observable toxicity. Three animals who received 14 mg/kg cisplatin and saline “protection” died after cisplatin injection. Four others exhibited lethargy for 24 hours but recovered without any further treatment. Animals receiving butyrate and cisplatin had an average weight gain of 106 ± 41 g; animals receiving saline and cisplatin had an average gain of 122 ± 35 g (*P* not significant, Student *t* test).

Histone Deacetylase Inhibitor Study Results

Figure 3 shows average threshold shift for animals who received sodium butyrate and those who received saline (control group). Across all frequencies, threshold shift was approximately 5 to 6 dB in the control animals. Sodium butyrate administration essentially eliminated this loss. The difference in threshold shift between the two groups was statistically significant (*P* = .001, two-way ANOVA).

DISCUSSION

Histone deacetylase inhibitors may be agents that are protective to auditory hair cells in vivo without compromising systemic cancer therapy. Some HDACis in clinical trials against human

cancers show promise as anti-tumor agents; they are well tolerated and have few side effects.⁸

These data demonstrate that sodium butyrate is also otoprotective. We hypothesized this otoprotective effect might exist based on the known neuroprotective effects of sodium butyrate. In this single-dose cisplatin model, 12 days of daily sodium butyrate (7 days before and 5 days after cisplatin) essentially eliminated the HL associated with cisplatin.

We chose DPOAEs as a measure of cochlear function, because they provide a sensitive assay of the functional state of outer hair cells; and cisplatin's major effect is dose-dependent injury to outer hair cells.¹²

There are several limitations to these data. In humans, cisplatin is typically given repeatedly at intervals of 2 to 4 weeks for several months. HL does not typically develop after a single cycle. Ideally, the effect of sodium butyrate would be studied in animals receiving cisplatin repeatedly over several months. We tested our hypothesis in a single-dose model to make the labor and cost of the experiment feasible.

Most workers have found great variability in the amount of cisplatin-induced threshold shift in guinea pigs.² We also found substantial variability both in the amount of damage produced by the single-dose model of cisplatin and in the amount of protection afforded by sodium butyrate. Cisplatin threshold shift ranged from -2 dB to -30 dB in unprotected animals. In the protected animals, the threshold shifts ranged from +10 dB (indicating improvement in hearing after the butyrate treatment) to -2 dB. Subtle health difference in individual animals and differences in cisplatin resistance may both play a role.^{2,13}

This study adds to the literature suggesting that insight into hair cells can be drawn from studies of neurons. In addition to the mechanical transduction apparatus at their apical ends, hair cells also possess synaptic machinery at their basal poles. It is plausible that they would share regulatory and protective mechanisms with neurons. For example, it has been shown that hair cells contain the neuron-specific splice variant of the gene RE-1 silencing transcription factor (REST), known to be an important determinant of neuronal fate.¹⁴ It is likely that further important insights about hair cell biology and function can be made by considering hair cells, at least in some respects, as analogous to neurons.

The mechanism of HDACi otoprotection is unclear but may be analogous to the mechanism of neuroprotection. Neuronal protection by HDACis may not be related to histone acetylation, but rather to acetylation of cytoplasmic proteins and subsequent upregulation of protective factors such as Sp-1.¹¹ New findings about the precise mechanism of HDACi protection in neurons should allow more targeted hypothesis generation and testing in oto-protective studies.

All normal cells have protective mechanisms, but it is energy-inefficient to keep protective machinery activated at all times. Thus, cells need rapid triggers to turn on protective machinery after stress. A cell under little or no stress, which is unexpectedly subjected to a major insult is particularly vulnerable because protective mechanisms will be at a nadir. Pharmacologically activation of protective mechanisms should be helpful in many forms of neurotoxicity and ototoxicity. Cisplatin-induced ototoxicity is a special case in that the ototoxic insult is "planned" and predictable. It should be possible to administer protective agents at precisely timed intervals before the insult. Cells have multiple protective pathways (e.g., the heat shock system¹⁵), and it may well be that the ideal protective regimen might be several agents that activate several different pathways.

CONCLUSIONS

The histone deacetylase inhibitor sodium butyrate protects against cisplatin-induced HL in guinea pigs. Because HDACis are tumoricidal as well as otoprotective, they may prove to be important in the clinical setting. Understanding the molecular mechanism of HDACi protection may help clarify the molecular basis of cisplatin-induced HL.

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Fig. 1.
Flow chart of the experimental design.

Fig. 2. Percent of guinea pigs with measurable hearing loss, and percent survival, at cisplatin doses of 8 (n = 5), 10 (n = 5), 12 (n = 10), 14 (n = 10), and 16 mg/kg (n = 5). Hearing loss and mortality after a single dose of cisplatin in guinea pigs.

Fig. 3.

Average threshold shifts ($\pm$ standard error of mean) from 3,500 to 20,000 Hz in animals who received cisplatin and sodium butyrate ($n = 17$ animals) compared with threshold shifts in control animals who received cisplatin and sham protection with saline injections ($n = 16$ animals). Threshold shifts were calculated for each ear and then averaged for each animal. Each animal is considered as one data point. Cisplatin caused an approximately 5-dB threshold shift across all frequencies; sodium butyrate essentially eliminated this shift.

TABLE I

Guinea Pig Use.

Study	Number of Animals
Excluded as a result of hearing loss or asymmetric hearing on initial testing	4
Butyrate toxicity study	5
Cisplatin toxicity study (8 mg/kg)	5
Cisplatin toxicity study (10 mg/kg)	5
Cisplatin toxicity study (12 mg/kg)	10
Cisplatin toxicity study (14 mg/kg)	10
Cisplatin toxicity study (16 mg/kg)	5
Cisplatin with butyrate protection	17
Cisplatin with sham (saline) protection	19 (3 died)
Total	80