Published in final edited form as:

J Neurochem. 2009 March ; 108(5): 1226–1236. doi:10.1111/j.1471-4159.2009.05871.x.

Aminoglycoside-induced histone deacetylation and hair cell death in the mouse cochlea

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Abstract

Post-translational modification of histones is an important form of chromatin regulation impacting transcriptional activation. Histone acetyltransferases (HATs), for example, acetylate lysine residues on histone tails enhancing gene transcription, while histone deacetylases (HDACs) remove those acetyl groups and repress gene transcription. Deficient histone acetylation is associated with pathologies, and histone deacetylase inhibitors have been studied in the treatment of cancer and neurodegenerative diseases.

Here we explore histone acetylation in cochlear sensory cells following a challenge with gentamicin, an aminoglycoside antibiotic known to cause loss of auditory hair cells and hearing. The addition of the drug to organotypic cultures of the mouse organ of Corti decreased the acetylation of histone core proteins (H2A AcK5, H2B AcK12, H3 AcK9, and H4 AcK8) followed by a loss of sensory cells. Protein levels of HDAC1, HDAC3 and HDAC4 were increased while the HATs CBP and p300 remained unchanged. We next hypothesized that protecting histone acetylation should prevent cell death and tested the effects of HDAC-inhibitors on the actions of gentamicin. Co-treatment with trichostatin-A maintained near-normal levels of acetylation of histone core proteins in cochlear outer hair cells and attenuated gentamicin-induced cell death. The addition of sodium butyrate also rescued hair cells from damage by gentamicin. The results are consistent with an involvement of deficient histone acetylation in aminoglycoside-induced hair cell death by and point to the potential value of HDAC-inhibitors in protection from the side effects of these drugs.

Introduction

Post-transcriptional modification of histones modulates gene transcription through effects on tertiary chromatin structure, differentially affecting the access of the transcriptional machinery to sections of DNA. The N-terminal tails of the core histones, including H2A, H2B, H3 and H4, contain specific amino acid sequences for acetylation, methylation, phosphorylation, ubiquitination and sumoylation (Sadri-Vakili and Cha, 2006). Histone acetyltransferases (HATs), for example, add acetyl groups to lysine residues, thereby facilitating the binding of transcription factors to nucleosomal DNA and activating

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Disclosure Statement

There are no conflicts of interest for any of the authors.

The data contained in the manuscript have not been previously published, have not been submitted elsewhere, and will not be submitted elsewhere while under review.

All experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals and animal care was supervised by the University of Michigan's Unit for Laboratory Animal Medicine.

All authors have reviewed the contents of the manuscript, approve of its contents, and validate the accuracy of the data.

transcription while histone deacetylases (HDACs) remove acetyl groups promoting chromatin condensation and reduced transcription (Marks et al., 2001). These reversible changes are implicated in a variety of biological functions such as transformation, differentiation, cell survival, and cell death (de Ruijter et al., 2003; Marks et al., 2004).

Epigenetic modifications including deficient histone acetylation have been observed in many progressive neurodegenerative conditions, for example, in Huntington's disease (Taylor and Fischbeck, 2002). Aberrations of the acetylation homeostasis mostly result from the loss of HATs like CREB binding protein (CBP) and p300, and restoration of the balance contributes to the amelioration of neurodegenerative diseases (Saha and Pahan, 2006). HDAC-inhibitors increase histone acetylation in hippocampal tissue *in vivo* and restore learning ability and memory to a mouse model of neurodegenerative disease (Fischer et al., 2007). They also diminish neuroinflammation and neuron apoptosis and improve neurological performance in a cerebral ischemia model in animals (Kim et al., 2007). Furthermore, HDAC-inhibitors are being investigated for cancer therapy based on their ability to influence the expression of 7–10% of transcribed genes in cancer cells and to induce cell cycle arrest and senescence (Xu et al., 2007).

Four classes of mammalian HDACs have been identified and classified based on homology to yeast HDACs (Morrison et al., 2006; Gao et al., 2002). Class I HDACs, including HDAC1, 2, 3, and 8, are nuclear proteins expressed in most tissues and cell types, and function as transcriptional repressors. Class II HDACs include HDAC 4, 5, 6, 7, 9, and 10, and function as a shuttle between cytoplasm and nuclei. Class III, sirtuins, requires NAD⁺ for its enzymatic activity and Class IV, HDAC 11, has conserved residues in the catalytic core region shared by class I and II. Commonly used HDAC inhibitors such as trichostatin-A efficiently block all class I and II HDACs.

A histone acetylation imbalance may also be involved in the “ototoxic” actions of aminoglycoside antibiotics. Treatment with aminoglycoside antibiotics can lead to auditory or vestibular deficits in 10 and 20% of patients, curtailing their clinical use (Forge and Schacht 2000). Despite their toxic side effects, drugs such as gentamicin, kanamycin or tobramycin are in use worldwide to combat serious gram-negative infections, as prophylaxis in cystic fibrosis, and in the treatment of tuberculosis. Emerging applications are associated with ability of the drugs to suppress premature stop codons and thus serve as potential therapeutics for a variety of genetic diseases in their native form or as synthetic ‘designer aminoglycosides’ (Hainrichson et al., 2008; Hermann, 2007).

Animal models have linked aminoglycoside ototoxicity to oxidative stress and the loss of hair cells, the sensory cells in the cochlea and the vestibular system (Forge and Schacht, 2000). Cochlear sensory cells include the inner hair cells and three rows of outer hair cells which are located in the auditory end organ, the organ of Corti. Inner hair cells are the primary sensory cells that convert the mechanical acoustic input into receptor potential and release of neurotransmitters, triggering action potentials that are carried to the brain by the spiral ganglion neurons. The major function of outer hair cells is to enhance the performance of the cochlea, particularly at low intensities of sound. Surrounding supporting cells include the inner and outer pillar, Deiters', Hensen and Claudius' cells, but their function remains largely unexplored.

Cell death pathways in ototoxic events are complex and involve necrotic and apoptotic caspase-dependent and caspase-independent pathways (Jiang et al., 2006b). Recent studies have also shown that treatment of mice with kanamycin increased the levels of phosphatidylinositol 4,5-bisphosphate and decreased the acetylation of histone H3 in the nuclei of outer hair cells (Jiang et al., 2006a). We now hypothesize that kanamycin-induced

hypo-acetylation of histones may be associated with aminoglycoside-induced hair cell loss and that consequently, restoration of histone acetylation with HDAC-inhibitors should rescue inner ear hair cells. The present study therefore investigates acetylation of histone core proteins in gentamicin-induced ototoxicity in the organotypic culture of organ of Corti and assesses the effects of HDAC-inhibitors.

2. Materials and Methods

Materials

Polyclonal rabbit antibodies against HDAC1, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, and against acetyl-histone H3 (Lys9), acetyl-histone H2A (Lys5), acetyl-histone H2B (Lys12), acetyl-histone H4 (Lys8), and against histone H2A, histone H3 as well as corresponding secondary antibodies, RIPA buffer (catalogue number: 9806) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Polyclonal rabbit anti-CBP NT and mouse anti-GAPDH were purchased from Millipore (Billerica, MA). Polyclonal rabbit anti-p300 (N-15) came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary antibody for Western blot detection of GAPDH was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA). Alexa Fluor 488 secondary antibody, Alexa Fluor 488 phalloidin and rhodamine phalloidin were purchased from Molecular Probes (Invitrogen, Eugene, OR) and trichostatin A from Calbiochem (EMD Chemicals Inc, Gibbstown, NJ). Gentamicin linked to Texas Red (GTTR) and Texas Red were the kind gifts of Dr. Peter Steyger (Oregon Health & Science University, Portland, OR). Type I rat tail collagen was obtained from BD biosciences (San Jose, CA). ECL Plus Western Blotting Detection Reagents were purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). All other reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Animals

Sexually mature male (6 weeks) and female (8 weeks) CBA/J mice were purchased from Harlan Sprague-Dawley Co. (Indianapolis, IN). The animals were maintained on a 12-h light/12-h dark schedule and had free access to water and a regular mouse diet (Purina 5025; Purina, St Louis, MO). For breeding, one male and two female mice were placed in one cage after 1 week of acclimation to their habitat. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA) and animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine (ULAM).

Organotypic cultures of postnatal organ of Corti

The organ culture procedures were modified from published reports (Zheng and Gao, 1996, Ding et al., 2002). In brief, mice of postnatal day 3 (p3) were euthanized after antisepsis with 70% ethanol, and the inner ears were removed. The cochleae were immersed in cold Hank's Balanced Salt Solution and the lateral wall tissues (stria vascularis and spiral ligament) and the auditory nerve bundle were dissected away leaving the organ of Corti and spiral ganglion neurons. The explants were placed onto a previously prepared culture dish: a 15- μ L drop of rat tail collagen solution was allowed to polymerize for approximately 15 min on a 35 mm dish, then 1 mL of serum-free medium, including Basal Medium Eagle (BME), 1% serum-free supplement (Gibco catalogue number 51500-056; Invitrogen, Carlsbad, CA), 1% bovine serum albumin, 5 mg/mL glucose and 10 U/mL penicillin G were added. The cochlear explants were incubated (37°C, 5% CO₂) for 4 h, then an additional 1 mL of culture medium was added to submerge the explants.

Treatment of organ culture explants

Explants were incubated for two days to allow for recovery from dissection stress before any procedures were begun. The explants were then divided into experimental groups, and the medium was exchanged for new media containing drugs under study and incubated for the desired time points. For the studies of protection, explants were matched: the explant from one ear of an animal received gentamicin only and the explant from the other ear was treated with gentamicin combined with a potential protective agent. Unless otherwise noted, working concentrations were 0.2 mM gentamicin or 6 mM sodium butyrate dissolved in the culture medium. Trichostatin A (TSA) was dissolved in 100% DMSO and stored at -20°C if necessary. Four μL of a 0.1 mM TSA solution were added to 2 mL of culture medium for final concentrations of 200 nM Trichostatin-A and 0.2% DMSO. Vehicle control incubations were carried out with 0.2% DMSO.

Hair cell counts

Explants were fixed with 4% paraformaldehyde overnight at 4°C and permeabilized for 30 min with 3% Triton X-100 in PBS at room temperature. The specimens were then washed three times with PBS and incubated with Alexa 488 phalloidin (1:200) or rhodamine-phalloidin (1:100) at room temperature for 30 min. After several rinses in PBS, the specimens were mounted on a slide with GEL/MOUNT^{TM} (Biomedica Corp., Foster City, CA). The phalloidin-stained stereociliary bundles and circumferential F-actin rings on the cuticular plate of outer and inner hair cells allowed the localization of cells that were present or missing. Cell populations were assessed on a Leitz Orthoplan upright microscope equipped for epifluorescence, using a 50x oil immersion objective. The right objective had a 0.17-mm calibrated scale imposed on the field for reference and the single row of inner hair cells and all three rows of outer hair cells were oriented longitudinally within each 0.17-mm frame. Each successive 0.17-mm field was evaluated for the absence of inner and outer hair cells beginning at the apex and moving down the organ of Corti to the base. Cell counts were entered into a computer program (KHRI Cytocochleogram, version 3.0.6) for comparison with a normative data base established from control specimens and the percentage of missing hair cells was calculated and plotted as a function of distance from the apical turn to the basal turn of the explant.

Immunofluorescent assessment of organ of Corti explants

Explants were fixed with 4% paraformaldehyde overnight at 4°C and permeabilized for 30 min with 3% Triton X-100 in PBS at room temperature. The specimens were then washed three times with PBS and blocked with 10% normal goat serum for 30 min at room temperature, followed by incubation with the primary antibodies against acetyl-histone H2A (Lys5), acetyl-histone H2B (Lys12), acetyl-histone H3 (Lys 9), acetyl-histone H4 (Lys 8), and polyclonal rabbit anti-HDAC1 diluted 1:50 in PBS at 4°C for 72 h. After washing three times with PBS, the secondary goat anti-rabbit antibody (Alexa 488-conjugated antibody) was applied at a concentration of 1:200 in PBS at 4°C overnight in darkness. For fluorescent visualization of hair cells, the specimens were incubated with rhodamine phalloidine (1:100) at room temperature for 1 h; for fluorescent visualization of nuclei, the specimens were incubated with Hoechst 33342 (2 $\mu\text{g}/\text{mL}$ in PBS) at room temperature for 30 min. After several rinses in PBS, the specimens were mounted on a slide with GEL/MOUNT^{TM} (Biomedica Corp., Foster City, CA). Preparations were observed with a Leica DMIL microscope (Leica Microsystems Inc. Bannockburn, IL) and imaged with an Olympus Fluoview Confocal Laser Scanning Microscope-FV500 (Olympus American, Melville, NY).

Confocal images of different preparations in a set of experiments were taken under identical conditions and equal setting parameters. The fluorescence intensity of "regions of interest" from each confocal image was measured and analyzed with ImageJ software (National

Institute of Health, Bethesda, MD). For the acetylation of histone core proteins, the pixel intensity of the outer and inner hair cell nuclei was measured in the basal segment of the explant. For gentamicin uptake, the pixel intensity of outer hair cell images from 0.212-mm segments of the basal, middle and apical regions was measured. Each segment contained about 70 outer hair cells and the average fluorescence intensity per cell was obtained. Relative fluorescence intensity was quantified by normalizing the ratio of average fluorescence intensity of treated cells to the average fluorescence of control cells. Individual preparations from three animals per condition were examined and data subjected to statistical analysis.

Extraction of total proteins and Western blotting

After incubation, explants were washed with phosphate-buffered saline (PBS) and 8 explants were pooled and homogenized in ice cold RIPA lysis buffer by using a glass/glass micro tissue grinder for 30 sec. Subsequently, tubes were sonicated in an ultrasonic water bath for 15 sec. After 30 min on ice, insoluble material was removed at 12,000 ×g at 4°C for 10 min and the supernatants were stored at -80°C until analysis.

Protein concentrations were determined using the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard, and samples of 40 µg each were separated by SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Pierce, Rockford, IL) and blocked with 5% non-fat dry milk in PBS with 0.1% Tween 20 (PBS-T). The membranes were incubated with primary antibodies rabbit anti-HDAC1 (1:1,000), anti-HDAC3 (1:1,000), anti-HDAC4 (1:1,000), anti-HDAC5 (1:1,000), anti-HDAC6 (1:1,000), anti-HDAC7 (1:1,000), anti-Histone H2A (1:1,000) and anti-Histone H3 (1:1,000), anti-CBP NT (1:3,000), and anti-p300 (1:300) or mouse anti-GAPDH (1:10,000) in 5% skim milk in PBS-T overnight at 4°C, and then washed three times (10 min each) with PBS-T. Membranes were incubated with an appropriate secondary antibody at a concentration of 1:1,000 (Cell Signaling Technology Inc., Danvers, MA), or 1:10,000 (Jackson Immunoresearch Laboratory, West Grove, PA) for 1 hr at room temperature. Following extensive washing of the membrane, the immunoreactive bands were visualized by enhanced chemiluminescence.

Scans of Western blots were analyzed using AlphaEase software SpotDenso tool (Alpha Innotech, San Leandro, CA). The band densities were first normalized to background, then the HDACs/GAPDH ratio was calculated from the band density of HDACs and GAPDH run on the same gel. Finally, the difference in relative densities of the control and experimental bands was tested for statistical significance using Primer of Biostatistics software (McGraw-Hill Software, New York, NY).

Gentamicin uptake

Explants were incubated with serum-free medium containing 1.56 µg/mL TR, 1.56 µg/mL GTTR (Myrdal et al., 2005), or GTTR plus 200 nM trichostatin-A for 8 h. After fixation and permeabilization as described for immunofluorescence of explants, the specimens were incubated with Alexa Fluor 488 phalloidin (1:100) for 1h and Hoechst 33342 (2 µg/mL in PBS) for 30 min for fluorescent visualization of hair cell F-actin and nuclei, respectively, and the assessment of gentamicin fluorescence.

Statistical analysis

Data were statistically evaluated by Student's t-test and by analyses of variance with Student-Newman-Keuls test for significance ($p < 0.05$) using Primer of Biostatistics software (McGraw-Hill Software, New York, NY). For the relative fluorescence intensity, a two

tailed one-sample t-test using GraphPad Software (GraphPad Software Inc., San Diego, CA) was applied.

3. Results

Gentamicin-induced hair cell loss in mouse organ of Corti organotypic cultures

Explants of the organ of Corti of the postnatal day 3 mouse, cultured for an additional 3 days (fig. 1A), were approximately 4.25 mm long and contained a total of about 2100 outer hair cells in three rows and 700 inner hair cells. Actions of gentamicin were mostly analyzed in basal segments, the primary location of its ototoxic action while middle and apical segments of the preparation were additionally evaluated for gentamicin uptake.

In control preparations, incubated for 24 h in culture medium, hair cells were well preserved (fig. 1B). With 0.2 mM gentamicin, the explants showed neither inner nor outer hair cell loss after 8 h (and 12 h, not shown) and only minimal loss in the basal segment at 16 h. Hair cell death became evident by 20 and 24 h but hair cells in the most apical area were spared. In contrast to its toxicity towards hair cells, gentamicin did not destroy non-sensory cells (Hensen's cells; fig. 1C) under these conditions.

Quantitative evaluation of surface preparations of the entire length of the organ of Corti confirmed that hair cell loss increased with increasing concentrations of gentamicin (fig. 2). A loss of 50% of hair cells was reached at a concentration of about 0.15 mM gentamicin.

Gentamicin reduces histone acetylation in outer hair cells and the histone deacetylase inhibitor trichostatin-A blocks this action

Acetylation of the histone core proteins H2A, H2B, H3, and H4 decreased significantly in both outer and inner hair cells after incubation with 0.2 mM gentamicin for 8 h and stayed at reduced levels by 16 and 20 h (fig. 3). The amount of total (acetylated plus non-acetylated) histone protein, measured by Western blotting for H2A and H3, remained unchanged by gentamicin treatment (data not shown). Trichostatin-A alone increased acetylation of all core histones in outer hair cells by 8 hr and maintained higher than normal levels through 20 hr. While the effect was significant for all histones, H2B and H4 showed considerably larger increases than H3 and H2A. When combined with gentamicin, trichostatin-A initially maintained acetylation of all core histones at near control levels, but its effect was diminished by 20 h in most cases. In contrast to hair cells, the acetylation of histones in Hensen cells did not change with gentamicin treatment. This was most evident in the 20 h-incubations where the staining for acetyl histones in Hensen's cell nuclei is seen above the rows of largely missing outer hair cells.

Gentamicin transiently increases histone deacetylases in outer hair cells

In order to probe into the reasons for the decreased histone acetylation, we determined protein levels of representative deacetylases HDAC1 and HDAC3 (class I HDAC), HDAC4, and HDAC6 (class II HDAC), and representative acetyltransferases CBP and p300 by Western blot (fig. 4A, C). Treatment of explants with 0.2 mM gentamicin for 8 h resulted in a significant elevation of HDAC1, HDAC3, and HDAC4, while HDAC6 remained stable. Add transferase. HDAC5 and HDAC7 were not detected under these conditions. Since the extracts from whole explants contain protein from sensory (hair) cells, supporting cells and nerve endings, immunocytochemistry was used to ascertain the cellular localization. Employing HDAC1 as a marker (fig. 4B) the transiently increased fluorescence intensity was found in the nuclei of outer hair cells.

Histone deacetylase inhibitors protect against gentamicin-induced hair cell loss

A single application of 200 nM trichostatin-A protected against gentamicin-induced loss of outer hair cells (fig. 5). In the base of the explant (fig. 5A), 0.2 mM gentamicin eliminated most outer hair cells while the additional presence of trichostatin-A preserved most cells in their normal appearance. Trichostatin by itself exhibited no toxicity at this concentration. In these experiments, 0.2% DMSO was added to control and gentamicin incubations as it was the solvent for trichostatin-A and a radical scavenger potentially interfering with gentamicin ototoxicity.

Quantitative evaluation of morphology along the entire length of the explant (fig. 5B) confirmed the base-to-apex gradient of gentamicin toxicity. Loss of both outer and inner hair cells was almost complete at the base while the apex was spared. DMSO, in agreement with previous reports (Sha and Schacht, 1998), afforded some protection against outer hair cell loss in the middle sections and against inner hair cell loss at the very base (fig. 5B, left panels). The additional presence of trichostatin attenuated the loss of both inner and outer hair cells from base to apex. Protection was more pronounced for outer hair cells than for inner hair cells. No loss of outer hair cells was apparent from the apex to the middle of the organ of Corti and a maximal 50% loss was seen in the base. Inner hair cell loss was attenuated uniformly from base to apex when compared to incubations with DMSO.

Sodium butyrate (6 mM), a water-soluble histone deacetylase inhibitor had a similar, albeit weaker, protective effect on gentamicin-induced hair cell loss (fig. 5B, right panels). Protection of outer hair cells was best in the apical and middle region and absent from the very base. Inner hair cell survival was minimally affected by sodium butyrate which alone did not cause any hair cell loss at this concentration.

Trichostatin-A does not interfere with gentamicin uptake into hair cells

Uptake of gentamicin into organ cultures was monitored with the drug tagged to Texas Red (GTTR). At 1.56 μ g GTTR/mL incubation medium, intracellular GTTR was evident. After 8 h of incubation (fig. 6A), tagged gentamicin was found in all outer and inner hair cells, but not in Hensen's cells. Trichostatin-A did not influence the amount of GTTR taken up by hair cells. Relative fluorescence intensity in outer hair cells calculated from basal, middle and apical segments showed no significant difference between GTTR and GTTR plus TSA. $N = 3$. Unconjugated Texas Red at comparable concentrations served as a control and did not enter the tissue.

4. Discussion

More commonly, loss of HAT activity rather than an increase in HDAC activity leads to acetylation imbalance (Saha and Palan, 2006). Transcriptional dysfunction

Histone deacetylation may also be involved in the ototoxicity of another drug, cisplatin, whose adverse effects can be attenuated by sodium butyrate (Drott et al., 2006).

Epigenetic changes, aberrant covalent modifications of DNA or histone core proteins, have increasingly become recognized as causes of or contributions to disease pathogenesis (Bhaumik et al., 2007; Lu et al., 2006). Histone deacetylation is linked to gene silencing and the development neurodegenerative disorders, and we propose here that it is involved in the death of inner ear sensory cells by aminoglycoside antibiotics.

The explant of the organ of Corti shows the preferential loss of outer hair cells in response to gentamicin and a base-to-apex progression that is characteristic of aminoglycoside damage to the cochlea in both human and experimental animals including the mouse (Wu et al.,

2001). Compared to the *in-vivo* condition, however, the time course of hair cell loss is accelerated, making explants an excellent model to probe mechanisms of drug ototoxicity in which hair cell death can be conveniently observed and manipulated (Zhang et al., 2003; Tabuchi et al., 2007). The fact that supporting cells such as Hensen's cells are spared also agrees with *in-vivo* observations and is best explained by the lack of uptake of gentamicin into these cells as shown in this study.

Under our experimental conditions, the gentamicin-induced demise of the sensory cells is evident within 16 to 24 hours after exposure to the drug. This time course is clearly not determined by the presence of gentamicin in hair cells as uptake can be observed as early as 30 min after the addition of the drug. Rather, we must assume that initial cell responses will resist the drug action by invoking survival pathways (Jiang et al., 2005) (reviewer disagree) until the continued exposure leads to a preponderance of cell death signaling. This delay is another analogy to aminoglycoside ototoxicity *in vivo* where the drugs can be initially present in hair cells without any measurable signs of pathology (Hiel et al., 1993).

The deacetylation of histone core proteins may be one of the triggers to cell death. It begins when hair cells are still intact and is seen in both outer and inner hair cells but not in Hensen cells which remain unaffected by gentamicin. The fact that all histone core proteins are affected suggests a global action of the drug, which can be best explained by the upregulation of several HDACs by gentamicin treatment. Although HDACs target histone protein substrates acting as transcription repressors to selectively alter gene expression they also target many non-histone substrates such as hormone receptors, chaperone proteins and cytoskeleton proteins, which regulate cell proliferation and cell death (Xu et al., 2007). This raises the question of the potential downstream consequences of gentamicin-induced histone deacetylation. Of the different types of HDACs tested here, HDAC6 preferentially targets non-histone substrates, including α tubulin and Hsp90 (Kawaguchi et al., 2003). The fact that gentamicin affected HDAC1, -3 and -4, but not HDAC6 suggests an interference with the acetylation of histone proteins that alter gene expression.

Compelling evidence for a causal relationship between histone deacetylation and cell death comes from the observation that the HDAC inhibitor trichostatin-A restores the acetylation imbalance with a consequent preservation of hair cells. This explanation is supported by the facts that trichostatin-A does not influence the uptake of gentamicin into cells (which could also lead to "protection") and that another HDAC inhibitor, sodium butyrate, likewise acts in a protective manner. The lower efficacy of sodium butyrate may reflect its inability to block class II HDACs of which at least HDAC4 is upregulated by gentamicin (reviewer disagree, because SB can also block class II such as HDAC4). We may state: Both TSA and SB showed protection from gentamicin-induced hair cell loss in both inner and outer hair cells with relative low efficacy of protection by SB. The low efficacy of protection by SB can be explained with generally accepted one concept of free radical theory involved in aminoglycoside-induced ototoxicity among many other factors. Since the solution of TSA, DMSO, is a free radical scavenger; this may also contribute to the protection provided by TSA. In addition, TSA and SB may have different sensitivity in inhibition of HDAC between (Wang et al., 1999). Both inhibitors are more efficient at preserving outer hair cells than inner hair cells, an intriguing observation that deserves further exploration.

Invoking histone deacetylation in aminoglycoside-induced sensory cell death has potential translational consequences. Several HDAC inhibitors have already been approved for clinical use and a large number are in clinical trials (Glaser, 2007). Such inhibitors could benefit the large population of patients that receive aminoglycoside antibiotics for emergency treatment, against tuberculosis, or as prophylaxis in cystic fibrosis (Forge and Schacht, 2000) and all of whom are at risk for irreversible hearing and balance deficits. The

observation that even dietary supplements can change HDAC activities (Dashwood and Ho, 2007) may make a protective treatment accessible to patients in developing countries where aminoglycoside use is widespread and expensive medications are not affordable.

Acknowledgments

This study was supported by research grant DC-03685 and core grant P30 DC-05188 from the National Institute on Deafness and Other Communication Disorders, NIH.

Abbreviations

DMSO	Dimethylsulfoxide
GTTR	Gentamicin linked to the Texas Red
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
PBS	Phosphate buffered saline
SB	Sodium butyrate
TR	Texas Red
TSA	Trichostatin-A

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Figure 1. Gentamicin-induced hair cell loss in mouse organotypic cultures of the organ of Corti

A: A whole mount of the organ of Corti explanted at postnatal day 3 and maintained in culture for 2 days was stained for F-actin with rhodamine phalloidin (red fluorescence). The outer stained perimeter contains the hair cell layer. Basal, middle and apical segments indicate the approximate areas analyzed in this study. Scale bar = 500 μ m.

B: Base-to-apex gradient of hair cell loss. Surface preparations of the basal segment show the outlines of inner and outer hair cells stained for F-actin with rhodamine phalloidin. Control incubations in the absence of gentamicin maintained a normal appearance at 24 h. In the basal segment of the organ of Corti, outer hair cell loss began after 16 h of incubation with gentamicin and progressed with increasing time while outer hair cells at the apex (GM 20h Apex) remained intact. The images are representative of 8 individual preparations at each time point. Scale bar = 20 μ m.

C: Hensen's cells remained intact after treatment with 0.2 mM gentamicin for 20h. Nuclei were stained blue with Hoechst 33342 and Hensen's cells can be identified by their position on the surface preparation and in comparison with F-actin staining for hair cells. Abbreviations: GM, gentamicin; IHC, inner hair cells; OHC1, OHC2, OHC3, outer hair cells of the first, second and third row, respectively.

Figure 2. Dose-dependence of outer hair cell loss

Surface preparations of the entire length of the explant were evaluated quantitatively for hair cell loss as described in 'Methods'. After 24 h of incubation the percentage of missing outer hair cells increases with increasing doses of gentamicin.

Percent of hair cells missing at gentamicin concentrations > 0.05 mM is significantly different ($P < 0.05$) from controls. Controls, $n = 7$; 0.05 mM, $n = 3$; 0.075 mM, $n = 5$; 0.1 mM, $n = 15$; 0.2 mM, $n = 9$. Data presented as average $\pm$ SD.

Figure 3 A-D. Acetylation of histone core proteins in both outer and inner hair cells is decreased by gentamicin and restored by trichostatin-A

Explants received the treatments as stated below and were stained (green) by immunocytochemistry for histone H3 AcK9 (A); histone H2A AcK5 (B); histone H2B AcK12 (C); or histone H4 AcK8 (D). Blue is Hoechst 33342 staining of nuclei. The figures are representative images of three individual preparations of the organ of Corti basal turn for each condition. Abbreviations: GM, gentamicin; vehicle, 0.2% DMSO; TSA, 200 nM trichostatin-A with 0.2% DMSO. IHC, inner hair cells; OHC1, outer hair cell first row; OHC2, outer hair cell second row; OHC3, outer hair cell third row. Scale bars = 20 μm. Left panels A, B, C, D: Explants were treated with 200 nM trichostatin-A for the times indicated on the images. 'Vehicle' denotes control incubation with 0.2% DMSO for 8 hr. Acetylation of histone is increased in the presence of trichostatin-A compared to the vehicle only.

Middle panels A, B, C, D: Explants were treated with 0.2 mM gentamicin for the times indicated on the images. Acetylation of all histones in both outer and inner hair cells decreased in a time-dependent manner as compared to an 8-hr incubation in control medium. Right panels A, B, C, D: Explants received both 0.2 mM gentamicin and 200 nM trichostatin-A for the times indicated on the images. Histone acetylation in both outer and inner hair cells is maintained at near control levels for the first 8 hr (16 hr in the case of H3) of incubation but decreases at later times.

Relative fluorescence intensity in both outer and inner hair cells obtained from the immunocytochemical images as described in the method section are stated at the bottom of each fluorescence image. Histones show similar patterns but different magnitude of decreased acetylation with gentamicin and increase with TSA at 8 h. Significance of differences: *: $P < 0.05$ vs. Control; **: $p < 0.01$ vs. Control.

Figure 4. Histone deacetylases are transiently up-regulated by gentamicin

4A: Western blots detected histone deacetylases 1, -3, -4 and -6 in homogenates of organ of Corti explants. Membranes were sequentially stained for HDAC1 and HDAC3 and for HDAC4 and HDAC6.

Significance of differences: *: $p < 0.05$, **: $p < 0.01$ compared to the corresponding control groups ($n = 3$, average $\pm$ SD).

4B: Immunocytochemistry of organ of Corti explants for histone deacetylase 1 (green) indicated a transient increase in outer hair cells at 8 h of gentamicin treatment. Blue is Hoechst 33342 staining for nuclei. The figure shows representative images from the basal turn of the organ of Corti ($n = 3$ at each time point). Scale bar = 20 μ m.

Abbreviations: GM, gentamicin; IHC, inner hair cell; OHC, outer hair cells; OHC1, outer hair cell first row; OHC2, outer hair cell second row; OHC3, outer hair cell third row; Hensen's, hensen's cell.

4C: Western blots detected histone acetylase CBP/p300 in homogenates of organ of Corti explants. Semi-quantitative analysis showed no difference between control and gentamicin treatment ($n = 3$, average $\pm$ SD).

Figure 5. Histone deacetylase inhibitors protect against gentamicin-induced hair cell loss

5A: Representative images from the basal turn of the organ of Corti after 20 h of incubation, showing the outline of hair cells stained with Alexa 488 phalloidin. Control: incubation with 0.2% DMSO; TSA: 200 nM trichostatin-A dissolved in 0.2% DMSO; GM 20h: explant treated with 0.2 mM gentamicin for 20 h; GM + vehicle: 0.2 mM gentamicin plus 0.2% DMSO; GM + TSA: 0.2 mM gentamicin plus 200 nM trichostatin-A dissolved in 0.2% DMSO. N = 8 for each condition. Scale bar = 20 μ m.

5B: Quantitative evaluation of loss of outer and inner hair cells along the entire length of the organ of Corti explant after 20 h of incubation with gentamicin in the absence or presence of 200 nM trichostatin-A or 6 mM sodium butyrate. There is no inner or outer hair cell loss in control incubations with sodium sulfate (0.5 mM) substituting for gentamicin sulfate or with DMSO alone. N = 8 for each condition. Abbreviations: GM, gentamicin; SB, sodium butyrate; TSA, trichostatin-A.

5C: Average of hair cell survival between groups of Gentamicin, gentamicin plus SB, gentamicin plus DMSO, and gentamicin plus both DMSO and TSA (n = 8, average $\pm$ SD, *p<0.05, **p<0.01).

Figure 6. Trichostatin-A does not affect gentamicin uptake into outer hair cells

Explants were incubated for 8 h with 1.56 $\mu\text{g/mL}$ gentamicin tagged with Texas Red (GTTR) in the absence (a) or presence (b) of 200 nM trichostatin-A. Red fluorescence is GTTR, green fluorescence is staining for F-actin in stereocilia of outer hair cells with Alexa 488-phalloidin, blue is Hoechst 33342 for nuclei. Images show a basal segment and are representative of three incubations under each condition. Scale bar = 20 μm .