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A molecular basis for human hypersensitivity to aminoglycoside antibiotics

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ABSTRACT

We have investigated the distribution of mitochondrial DNA polymorphisms in a rare maternally transmitted genetic trait that causes hypersensitivity to aminoglycoside antibiotics, in the hope that a characterization of its molecular basis might provide a molecular and cellular understanding of aminoglycoside-induced deafness (AGD). Here we report that the frequency of a particular mitochondrial DNA polymorphism, 1555^G, is associated non-randomly with aminoglycoside-induced deafness in two Japanese pedigrees, bringing the frequency of this polymorphism to 5 occurrences in 5 pedigrees of AGD, and in 4 of 78 sporadic cases in which deafness was thought to be the result of aminoglycoside exposure; both frequencies are significantly different from the occurrence of this mutation in the hearing population, which was 0 in 414 individuals surveyed. The 1555^G polymorphism occurred in none of 34 aminoglycoside-resistant individuals. We propose a specific molecular mechanism for aminoglycoside hypersensitivity in individuals carrying the 1555^G polymorphism, based on the three-dimensional structure of the ribosome, in which the 1555^G polymorphism favors aminoglycoside binding sterically, by increasing access to the the ribosome cleft.

INTRODUCTION

Although it has been known for more than 50 years that therapeutic aminoglycoside antibiotics have the side affects of sensorineural deafness, as well as vestibular toxicity and nephrotoxicity (1, 2), the molecular mechanism of this toxicity has remained obscure, even though aminoglycoside treatment has been the method of choice for experimentally inducing sensorineural deafness in animals (3).

We recently proposed that the ototoxic site of action of aminoglycoside antibiotics might be the mitochondrial ribosome, in light of several known facts. First, a rare trait for hypersensitivity to aminoglycoside antibiotics was known to be transmitted maternally, consistent with its transmission on the mitochondrial genome (4, 5). Second, every known mitochondrial genetic disease has sensorineural hearing loss or deafness as a common phenotype, as if sensory hair cells of the inner ear have a critical requirement for mitochondrial function (6, 7). Third, mitochondrial ribosomes share more similarities to bacterial ribosomes (the site of action of aminoglycoside antibiotics) than either do with the mammalian cytosolic ribosomes (8–10). Fourth, human tissue culture studies had shown that resistance of mammalian cells to antibiotics directed at the prokaryotic ribosome occurs by mutation of the 12S or 16S rRNA genes of the mtDNA (11–13). Fifth, ultrastructural studies of aminoglycoside poisoned hair cells of the inner ear had shown that mitochondrial dysmorphology of hair cells is one of the earliest observable phenotypes of aminoglycoside poisoning (3).

For these and other reasons we previously investigated sequence variation of 12S and 16S rRNA genes in three Chinese pedigrees with AGD carried on the mitochondrial DNA, who share three polymorphisms in the 12S and 16S rRNA (14). In order to address the issue of how often these polymorphisms are associated with AGD we have investigated the frequency of their occurrence in two Japanese AGD pedigrees and 78 sporadic cases of AGD from a Chinese hospital. The frequencies of these same three polymorphisms were also investigated in a study population of 34 aminoglycoside-resistant individuals from the same hospital who received normal doses of aminoglycosides and experienced no hearing loss.

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MATERIALS AND METHODS

DNA samples from Japanese AGD pedigrees, Chinese sporadic AGD cases, aminoglycoside-resistant cases and normal hearing individuals

Hair samples from two separate pedigrees (Figure 1) from Akita, Japan were obtained. Blood and hair samples from sporadic cases of AGD, as well as normal hearing people, were from the Tiedao Medical College. The patients are listed below giving their patient number, sex (m or f), age (in years), aminoglycoside(s) received (s=streptomycin, g=gentamicin, k=kanamycin, j=amikacin) and days treated. nd= not determined.

Sporadic cases: 1 f,1,s,7; 2 m,1,s,7; 3 m,5,g,6; 4 m,0.5,g,10; 5 f,5.5,g,6; 6 m,1,g,8; 7 f,2,g,10; 8 f,1.08,g,7; 9 m,6,g,k,7; 10 m,1,g,9; 11 f,4,g,4; 12 m,1,g,k,7; 13 m,1,g,5; 14 m,2.5,k,5; 15 f,1,g,6; 16 f,4,g,5; 17 m,7,k,7; 18 m,1.3,k,g,s,30; 19 m,22,s,15; 20 m,1,g,k,10; 21 f,3,g,10; 22 f,8,g,60; 23 m,0.58,g,11; 24 f,1.5,g,8; 25 f,1.6,g,7; 26 m,1,g,5; 27 m,0.5,g,10; 28 f,1,g,7; 29 f,4,g,nd; 30 f,0.66,k,7; 31 f,1,g,s,7; 32 m,10,g,5; 33 m,5,k,7; 34 f,1,g,15; 35 f,0.83,k,7; 36 m,3,g,k,8; 37 m,3,g,k,8; 38 m,1,s,7; 39 m,2,g,6; 40 f,1,s,g,7; 41 m,7,g,6; 42 f,4,s,7; 43 m,0.5,g,7; 44 m,2,k,5; 45 m,1.5,k,6; 46 f,0.58,g,k,10; 47 m,5,g,7; 48 f,2,k,5; 49 m,2,g,k,7; 50 f,5,g,6; 51 f,0.58,k,6; 52 m,7,g,8; 53 f,1,g,k,5; 54 m,2.5,g,7; 55 f,3,k,6; 56 m,7,g,s,7; 57 nd; 58 f,2,k,7; 59 f,9,g,6; 60 m,1,g,7; 61 f,11,g,11; 62 m,8,g,9; 63 f,8,g,8; 64 f,8,g,8; 65 m,6,g,10; 66 m,10,g,7; 67 f,10,g,9; 68 m,14,g,7; 70 m,14,k,7; 71 m,8,g,7; 72 m,11,g,6; 73 f,4,g,6; 74 m,6,g,7; 75 m,9,g,6; 76 m,10,g,7; 79 m,8,k,7; 80 f,7,g,6; 82 nd.

Aminoglycoside-resistant cases: 111 f,48,j,k,8; 112 m,61,j,k,17; 113 m,62,j,k,8; 114 m,73,g/k,9/15; 115 m 70,j,k,9; 116 m,60,j,k,21; 117 m,64,j,k,14; 118 m,27,j,k,14; 119 m,72,j,k,10; 120 f,46,j,k,11; 121 f,65,j,k,15; 122 f,36,g,s,7; 123 m,58,s,90; 124 f,20,g,k,15; 125 f,22,s,g,5; 126 m,22,g,6; 127 m,22,s,90; 128 m,23,s,nd; 129 m,22,g,7; 130 m,23,g,k,45; 131 f,22,s,nd; 132 f,35,s,60; 133 f,19,s,10; 134 f,32,j,9; 135 f,58,j,16; 136 f,73,j,10; 137 m,77,j,7; 138 m,61,j,10; 139 f,64,j,7; 140 m,71,j,11.

DNA samples from 104 normal individuals of Chinese descent were provided by M.Stoneking and N.Saha.

DNA isolation, PCR amplification conditions, primers and restriction digestion

DNA was extracted from 1μl of blood by the addition of 20μl of Genereleaser and the manufacturers suggestions followed (Bioventures Inc., Murfreesboro, TN). DNA was extracted from a single hair using Chelex 100 (Bio Rad Laboratories Inc, Hercules, CA) following the method of Singer-Sam and Tanguay (15). PCR reagents were added directly to these extracts and DNA amplified, with hemi-nesting, in two sets of 20 cycles using Taq polymerase (Perkin-Elmer Corp., Norwalk, CT).

Primers were named for their approximate binding site in the human mtDNA genome and are written 5′ to 3′ following the Cambridge numbering system (16). Primers used in the detection of the 1555 polymorphism were h1.48 (bases 1525−1549), h1.52 (bases 1525−1549), h1.56 (bases 1589−1566) and h1.60 (bases 1624−1601). Reaction conditions were 94°C, 30 sec; 60°C, 20 sec. The 1555 A to G mutation results in the loss of the *Bsm*A I restriction site in the PCR product.

The 663 polymorphism was detected with primers h0.59 (bases 591−611), h0.63 (bases 632−652) and h0.72 (bases 748−728). Reaction conditions were 94°C, 30 sec; 57°C, 20 sec. The 663 A to G mutation creates a *Hae* III restriction site in the PCR product.

The 1736 polymorphism was amplified with primers h1.48, h1.52 and h1.73 (bases 1757−1737). This last primer contains a mismatch at base 1739 (G for A) thereby creating a restriction site for *Mbo* I when base 1736 is a G. Reaction conditions were 94°C, 30 sec; 50°C, 20 sec; 72°C, 20 sec.

All restriction endonucleases were purchased from New England Biolabs. Quantitative digestion of an aliquot from the PCR reaction was carried out using the appropriate buffer and temperature. Restriction digests were analyzed on NuSieve agarose (3%) or polyacrylamide (12%) gels.

DNA sequence analysis

Double stranded PCR product was purified with a Centricon 10 (Amicon, Beverly, MA). Sequencing was carried out using the Sequenase Version 2.0 kit (USB, Cleveland, OH) following their instructions with the following modifications. After the DNA was heat denatured it was annealed on ice with primer h1.56. The labeling reaction was carried out for 45 seconds using a 1:200 dilution of the labeling mix.

Computer modeling

Structures of wild type and mutated human 12S rRNA were generated with two short molecular dynamics simulations. Calculations were performed on a 20 base RNA fragment comprising two strands; 5′ CACCCUCCUC and 3′ AACAGAGGAG (in the wild type) with G replacing A in the mutant. In each case the underlined bases (including the A−C mismatch in the wild type) were initially positioned in a base paired duplex conformation with the three remaining bases of each strand in a random coil conformation. The structures were then observed after 3.6 picoseconds of molecular dynamics analysis.

RESULTS

Occurrence of the 1555^G, 1736^G and 663^G polymorphisms among Japanese deafness pedigrees

It had recently been reported by Hu *et al.* and Higashi that the aminoglycoside-induced deafness (AGD) trait was maternally transmitted (4, 5). We investigated mtDNA sequence variation

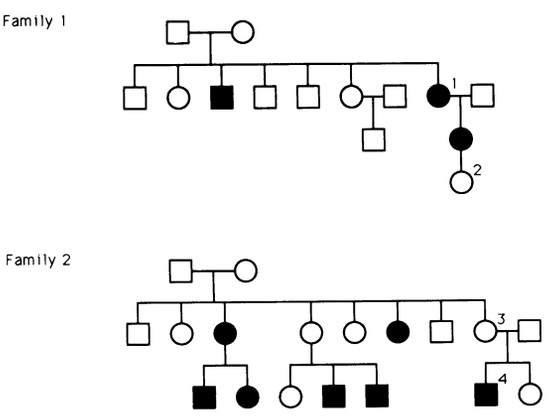

Figure 1. Japanese pedigrees with maternally transmitted aminoglycoside otosensitivity. Solid symbols represent individuals who lost their hearing after receiving aminoglycosides. Numbers indicate the individuals studied in this work.

from multiple individuals of two separate Japanese AGD pedigrees studied by Higashi (Figure 1), specifically for the 1555^G, 1736^G and 663^G mtDNA sequence variations which occur together among 3 Chinese AGD pedigrees (14). Both pedigrees shared the rare 1555^G polymorphism (Figures 2 and 3, Table 1), which was present in 0/414 hearing controls, of which 274 were Asian. One of the pedigrees was also found to have both the 1736^G and 663^G polymorphisms whereas the other pedigree had neither.

Occurrence of 1555^G, 1736^G and 663^G among sporadic deafness cases suspected to be the result of aminoglycoside exposure

If the mtDNA genetic variant 1555^G which we have now found in 5 of 5 AGD pedigrees (100%) is indeed an important contributor to AGD, one might expect to find the same variant in sporadic cases of deafness which occurred subsequent to aminoglycoside treatment. In our investigation of the occurrence of three polymorphisms at positions 1555, 1736 and 663, the 1555^G variant occurred in 4 of 78 (5%) such cases (Figure 2, Table 1). None of these 4 patients had either the 1736^G nor 663^G polymorphisms. Four other patients (5%) were found to possess both 1736^G and 663^G, however, these two polymorphisms were also found, usually together, in normal hearing controls (8% and 9% respectively, see Table 1).

Occurrence of 1555^G, 1736^G and 663^G among aminoglycoside-resistant individuals

Of the hearing individuals studied, 34 were known to have been administered aminoglycosides with no loss of hearing. None of these 34 patients carried the 1555^G polymorphism, although 9 did have both 1736^G and 663^G (Table 1).

Statistical analysis of 1555^G frequency in normal hearing individuals, pedigrees and sporadic deafness cases

One can compute the 99% confidence interval for the real frequency of 1555^G based on the observation of 5 occurrences in 5 trials. The 99% boundaries are 3.98 to 5.0. The 99% confidence interval for the real frequency of 1555^G in the normal hearing population (given zero occurrences in 414 trials) is bounded by 0.011 and zero. Hence there is no overlap at the 99% confidence limit of the estimates of 1555^G frequency in AGD pedigrees compared with the hearing population. In other words, there is a statistically significant difference in the frequency with which 1555^G occurs in AGD pedigrees versus the hearing population.

One can also evaluate whether the frequency of 1555^G occurrence in sporadic deafness cases is significantly different from that in the hearing population. If we assume, based on our population study of hearing persons presented above, that the 1555^G occurs at a frequency of 0.011 or less, then in a sample size of 78, we would expect a mean frequency of $(0.011)(78) = 0.858$ or less hearing individuals to carry the 1555^G polymorphism. However, we found 4 such individuals in 78 sporadic AGD cases, and based on Poisson assumptions, the probability of finding 4 such individuals given a mean frequency of 0.858 is less than 0.013. Thus we can be 99% certain that the frequency of 1555^G in the sporadic deafness cases is different than in the normal hearing population.

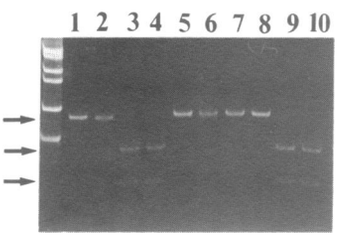

Figure 2. Detection of the 1555^G polymorphism by restriction digestion. A short region of the mt 12S ribosomal gene was amplified in a nested PCR reaction using primers h1.48+ h1.60 and h1.48+ h1.56. PCR products were digested with *Bsm*A I and the products separated on a 12% acrylamide gel. Arrow heads show the undigested 101 bp product, and the two fragments of the digested product. Lanes 1 and 2 are Japanese pedigrees with AGD, lanes 3 and 4 are normal hearing Japanese, lanes 5–8 are four cases of sporadic AGD, lanes 9–10 are aminoglycoside-resistant individuals. On the left are ϕX174 *Hae* III size markers.

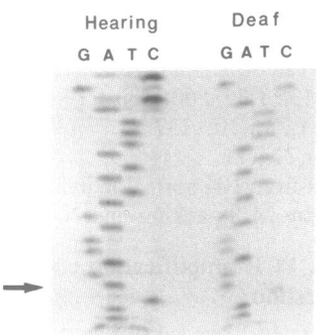

Figure 3. DNA sequence analysis of the 1555 polymorphism. PCR products, as in figure 2, were sequenced using primer h1.56 and the complementary strand is shown here from an aminoglycoside-resistant individual (left) and an AGD patient from one of the Japanese pedigrees (right).

Table 1. Association of mtDNA polymorphic sites with AG-induced deafness

mtDNA	Position	Occurrence in			
		Hearing individuals	AG-deaf pedigrees	AG-deaf sporadic	AG-resistant individuals
12S rNA	1555 G	0/274	5/5	4/78	0/34
	663 G	23/251	4/5	4/78*	9/34
16S rRNA	1736 G	22/266	4/5	4/78*	9/34

This table combines data from this study and the Asian persons from Prezant *et al.* (14). The 4 sporadic cases marked * are different individuals than the the 4 with 1555^G. AG resistant individuals are those who received aminoglycosides with no ototoxic side effects.

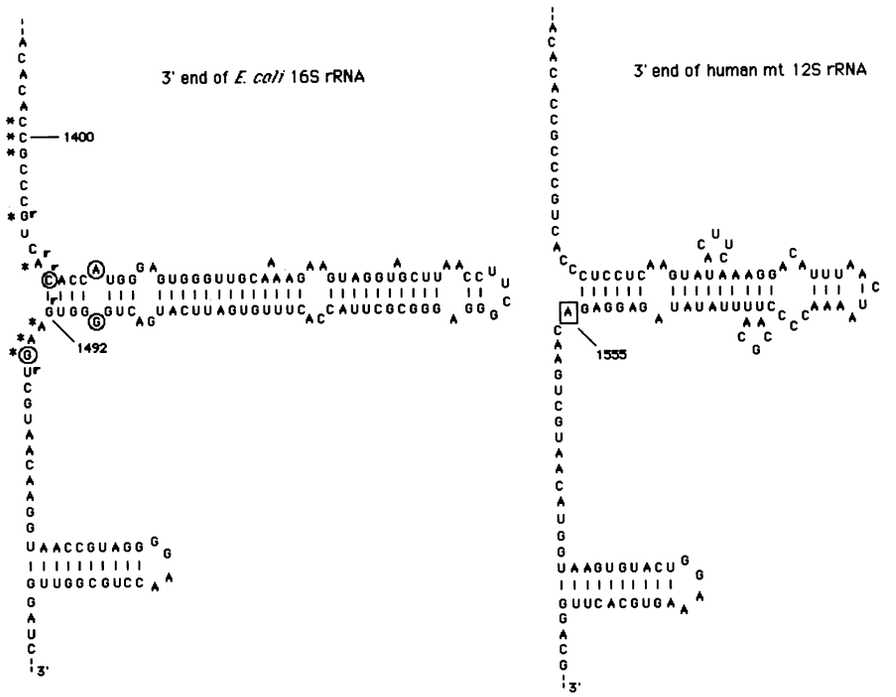

Figure 4. Comparison of 3′ ends of small rRNA from human mitochondria and *E.coli*. The secondary structures of the 3′ end of E.coli 16S rRNA (left) and human mitochondrial 12S rRNA (right) are shown. Bases protected by aminoglycosides are circled, bases conferring AG resistance, either by mutation or methylation, are marked **r**. Bases which interact with tRNA are marked *. The 1555 position on the 12S mt rRNA (boxed) is shown here as in the wild type, i.e. A, which is mutated to a G in some AGD individuals.

DISCUSSION

Non-random association of the mtDNA 1555^G polymorphism with aminoglycoside-induced deafness

The 1555^G polymorphism never occurred in 414 hearing people studied, of which 274 were Asian. Yet this rare polymorphism occurred in all 5 AGD pedigrees studied, three of Chinese and two of Japanese origin. The presence of 1555^G in some sporadic AGD cases strengthens the correlation between sporadic AGD and familial AGD. In sporadic cases in which family history is not available, diagnosis that deafness was the direct result of a genetic susceptibility to aminoglycosides is of course complicated by several factors. In some cases, patients may have received overdoses of aminoglycosides, or aminoglycoside toxicity may have been aggravated by the simultaneous administration of furosemide, ethnacrynic acid, or cisplatin. Such events could explain some of the other 74 sporadic cases which did not carry 1555^G. A more intriguing explanation is that there may be other mtDNA mutations in this group besides the 1555^G which predispose these individuals to aminoglycoside ototoxicity.

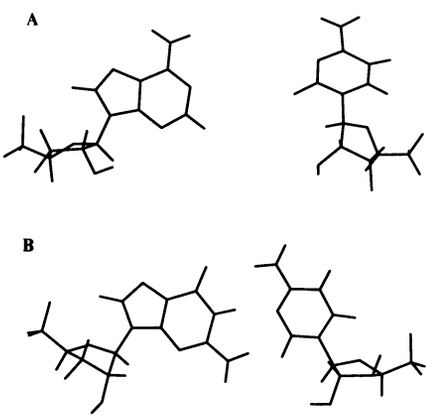

Figure 5. Molecular dynamics analysis of the effect of the 1555^G polymorphism on space in the ribosomal cleft. This computer generated image shows the predicted positions of base 1494^C and its wild-type partner 1555^A (**A**), compared with that of the mutant partner 1555^G (**B**).

The 1736^G and 663^G polymorphisms, which occurred with the 1555^G polymorphism in our earlier study of 3 Chinese pedigrees, are decoupled from 1555^G in a Japanese pedigree of AGD, and are thus probably not important for the AGD phenotype

In our previous study of the association of mtDNA polymorphisms with AGD, we found that the 1736^G and 663^G polymorphisms occurred in 3 of 3 Chinese AGD pedigrees, along with the 1555^G polymorphism (14). From this it could have been inferred that 1555^G, 1736^G and 663^G need to occur together in order to cause AGD. We speculated in that case that since the 1736^G and 663^G polymorphisms are also present in the hearing Asian population, 22 of 266 (8%) for 1736^G and 23 of 251 (9%) for 663^G, both were less likely to cause the AGD phenotype, but it was not possible to eliminate the hypothesis that the combination of 1736^G, 663^G and 1555^G polymorphisms was necessary for the AGD phenotype. That hypothesis can now be eliminated because 1555^G is decoupled from 1736^G and 663^G

in one of the Japanese pedigrees with AGD. The 1555^G polymorphisms also occurs uniquely in 4 of the sporadic AGD cases.

Strong confirmation of the notion that $1736^G$and 663^G are not important for the AGD phenotype is also provided by the fact that 27% of the aminoglycoside-resistant individuals carried both these polymorphisms (Table 1).

The 1555^G site which confers hypersensitivity to aminoglycosides is at a functionally important site of the ribosome from comparative studies in bacteria

Aminoglycoside antibiotics exert their antibacterial function by inhibiting or inducing errors in protein synthesis at the site of the bacterial ribosome, via interactions with specific sites of the bacterial ribosomal RNAs (17–19). Sites in ribosomal rRNA protected by aminoglycosides such as streptomycin, paromomycin, and hygromycin have been mapped (17, 18) and generally lie in highly conserved regions of the rRNA. The conservation of the sequence and structure of the mitochondrial small ribosomal RNA subunit allows one to compare it with the extensively studied *E.coli* 16S rRNA. Aminoglycosides bind primarily at, or near to, two regions of the *E.coli* 16S rRNA; the penultimate stem (i.e. positions 1409 and 1491 in the *E.coli* numbering system) and positions 911–915. These two regions are brought together in the secondary and tertiary structure of the 16S rRNA, surrounding the decoding site in the cleft of the ribosome (20–23). The 1555^G mutation occurs at the base of this penultimate stem (see Figure 4) and is presumed to introduce a new base-pair at the base of that penultimate helix. The importance of the penultimate stem in ribosomal function is evident from several lines of work (see Figure 4). First, a disruption of the base pairing at the base of this stem (positions 1401 or 1491) results in resistance to several aminoglycosides such as paromomycin and neomycin (24) as well as altering the proofreading ability of the ribosome (25). Second, it is adjacent to the decoding site with which the tRNA interacts directly, including bases 1492 and 1493 which lie at the base of the stem (i.e. equivalent to the 1555 position in human mt 12S rRNA) (26). Third, disruption of this stem greatly reduces ribosomal activity (27). Finally, the transition between active and inactive states of the ribosome appears to involve a conformational change in the structure of this stem (28).

Consideration of molecular dynamics allows a steric explanation for aminoglycoside binding to be proposed

The 1555^G mutation is inferred to introduce a new base pair at the stem of the penultimate helix. One might expect base-paired ribonucleotides to take up less volume at the aminoglycoside binding site. This expectation was tested by molecular dynamics analysis as described in materials and methods. We found by molecular dynamics analysis that the wild type bases tend to occupy a larger volume than the paired bases (Figure 5). Thus we infer that the 1555^G mutation will introduce a new base pair and that the pairing of bases 1494 and 1555 may reduce the volume taken up by the normally unpaired bases. This predicted reduction in volume may facilitate aminoglycoside binding either thermodynamically or kinetically.

Implications for a mechanism of aminoglycoside-induced hair cell death

When given in massive doses, aminoglycosides cause deafness in all mammalian species tested (29), primarily by poisoning hair cells. The question remains by what mechanism this death of hair cells proceeds. We propose that hair cell death in non-hypersensitive individuals is ultimately the result of inhibition or mis-translation at the level of mitochondrial protein synthesis and suggest a cellular mechanism for hair cell death below.

Since all mitochondrial proteins are involved in ATP production, an interference of mitochondrial protein synthesis would likely lead to a fall in ATP production. Ionic gradients within the ear are maintained by ATP-driven pumps, therefore a reduction in ATP levels in the cochlea might result in an imbalance in ionic concentrations in the stria vascularis, endolymph or the hair cells themselves. Eventually, intracellular accumulation of toxic levels of ions such as Ca^{2+} might lead to hair cell death by a mechanism such as excitotoxicity (30–32). The molecular and cellular model for aminoglycoside ototoxicity proposed is testable at many levels.

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