Objective: To investigate possible ototoxic effects of topical azithromycin (AZ) in the guinea pig.

Design: A prospective, controlled animal study.

Setting: The University of Texas Southwestern Medical Center at Dallas.

Participants: Twenty-three pigmented guinea pigs were given single, unilateral middle ear applications of a solution containing 3% (n=3), 2% (n=5), 1% (n=5), or 0.5% (n=5) AZ or saline (n=5). The contralateral ear served as the untreated control.

Main Outcome Measures: The animals were observed for behavioral changes for 2 weeks and then humanely killed. The ears were processed for anatomical evaluation. Morphologic changes were analyzed by quantitation of middle ear changes and cochlear inner and outer hair cell loss. Statistical analysis was performed to examine effects by dose.

Results: Analysis revealed extensive middle and inner ear changes associated with all formulations of AZ. Moderate correlation was found between the extent of middle ear changes and AZ concentration ($r^2=0.59$), whereas a strong correlation was seen between inner ear damage and AZ concentration ($r^2=0.94$). Both inner and outer hair cells were affected, with inner hair cell damage consistently greater than outer hair cell damage.

Conclusions: The results of this study demonstrate that ototopical AZ can cause middle ear changes and significant hair cell loss in the guinea pig. This finding, together with previous clinical reports, indicates that topical AZ should be used with caution in the clinical setting.


Author Affiliations:
Departments of Otolaryngology–Head and Neck Surgery (Drs Pawlowski, Wright, and Roland and Ms Koulich) and Biomedical Engineering (Dr Pawlowski), The University of Texas Southwestern Medical Center at Dallas; School of Behavioral and Brain Sciences, The University of Texas at Dallas (Drs Pawlowski and Wright); and InSite Vision Inc, Alameda, California (Drs Si and Hosseini).
taneously, on the day of surgery. For drug application, the animals were anesthetized with isoflurane inhalation; a small opening was made in the left tympanic bulla in an area ventrocaudal to the pinna, and 250 μL of test solution was carefully and slowly instilled into the left middle ear through the opening. The right ear served as the untreated control. An AZ solution of 3% (n=3), 2% (n=5), 1% (n=5), or 0.5% (n=5) or saline (n=5) was given in the left ear. The skin was then closed, and the animals were allowed to recover from the anesthesia. The animals were monitored daily for signs of distress, vestibular upset, or wound infection. Two weeks after surgery, the animals were humanely killed and both temporal bones were harvested. All animal procedures were approved by the UT Southwestern Institutional Animal Care and Use Committee.

The temporal bones were divided into medial and lateral portions and photographed using a surgical microscope and digital camera. The inner ear of each temporal bone was then perfused with 2.5% glutaraldehyde in phosphate-buffered saline and fixed in AZ-treated ears vs controls. In addition, the extent of change was compared between groups to determine if there was a statistically significant difference between the conditions of the middle ears.

### Table 1. Definitions for Severity of Middle Ear Damage

<table>
<thead>
<tr>
<th>Score</th>
<th>Mucosal Thickening</th>
<th>Hematoma</th>
<th>Hyperemia</th>
<th>Adhesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Similar to normal</td>
<td>Slight amount, ie, 1 large or 2 small blood clots</td>
<td>Slight amount, ie, 1 or 2 dilated, blood-filled vessels</td>
<td>Slight amount, ie, 1 or 2 small adhesions</td>
</tr>
<tr>
<td>2</td>
<td>Slight amount, ie, 1 or 2 spots of thickening</td>
<td>Moderate amount, ie, several small clots, with or without large clots</td>
<td>Moderate amount, ie, several dilated, blood-filled vessels</td>
<td>Moderate amount, ie, several small adhesions</td>
</tr>
<tr>
<td>3</td>
<td>Moderate amount, ie, several small areas of thickening</td>
<td>Extensive amount, ie, clots fill approximately 75% of surface area</td>
<td>Extensive amount, ie, dilated, blood-filled vessels in approximately 75% of surface area</td>
<td>Extensive amount, ie, adhesions fill approximately 75% of middle ear space</td>
</tr>
<tr>
<td>4</td>
<td>Extensive amount, ie, thickened mucosa in approximately 75% of surface area</td>
<td>Severe amount, ie, &gt;90% of surface area is occupied by clots</td>
<td>Severe amount, ie, &gt;90% of surface area filled by dilated, blood-filled vessels</td>
<td>Severe amount, ie, &gt;90% of middle ear space is occupied by adhesions</td>
</tr>
</tbody>
</table>

### Table 2. Definitions for Severity of Hair Cell Damage

<table>
<thead>
<tr>
<th>Score</th>
<th>Inner Hair Cell Region</th>
<th>Outer Hair Cell Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90%-100% of the hair and supporting cells in the segment are present and appear normal</td>
<td>90%-100% of the hair and supporting cells in the segment are present and appear normal</td>
</tr>
<tr>
<td>2</td>
<td>75%-89% of the hair cells and all supporting cells in the segment are present and appear normal</td>
<td>75%-89% of the hair cells and all supporting cells in the segment are present and appear normal</td>
</tr>
<tr>
<td>3</td>
<td>50%-74% of the hair cells and all supporting cells in the segment are present and appear normal</td>
<td>50%-74% of the hair cells and all supporting cells in the segment are present and appear normal</td>
</tr>
<tr>
<td>4</td>
<td>25%-49% of the hair cells and all supporting cells in the segment are present and appear normal</td>
<td>25%-49% of the hair cells in the segment are present and appear normal</td>
</tr>
<tr>
<td>5</td>
<td>Less than 25% of the hair cells and all supporting cells in the segment are present and appear normal</td>
<td>No hair cells or supporting cells remain along most (75%) of the segment; the basilar membrane is covered with cuboidal shaped cells</td>
</tr>
</tbody>
</table>

A score for the other 3 indices. The mean (SD) scores for mucosal thickening, hyperemia, and hematomas were added to the score for mucosal adhesions to give each ear a cumulative score for middle ear changes. Mean (SD) cumulative scores were compared between groups to determine if there was a statistically significant difference between the conditions of the middle ears in AZ-treated ears vs controls. In addition, the extent of change with increasing azithromycin concentration was analyzed using linear regression to determine if the damage correlated with dose.

### MIDDLE EAR SCORING

To determine the extent of damage, micrographs of the middle ear were analyzed and quantitated by 2 examiners (K.S.P. and C.G.W.) in a blinded manner. Each examiner used a table with values representing the specific degree of damage observed (Table 1). Scores were reported for 4 indices: mucosal thickening, hyperemia, hematomas, and mucosal adhesions. Because adhesions tend to span the middle ear space, only 1 score for adhesions was given per ear. To account for variations in response in different areas of the middle ear mucosa, each ear was divided into 4 quadrants (dorsal lateral, dorsal medial, ventral lateral, and ventral medial), and each quadrant was given a score for the other 3 indices. The mean (SD) scores for mucosal thickening, hyperemia, and hematomas were added to the score for mucosal adhesions to give each ear a cumulative score for middle ear changes. Mean (SD) cumulative scores were compared between groups to determine if there was a statistically significant difference between the conditions of the middle ears in AZ-treated ears vs controls. In addition, the extent of change with increasing azithromycin concentration was analyzed using linear regression to determine if the damage correlated with dose.

The indices used in this study have been used previously to describe middle ear changes, but, to our knowledge, this is the first time they have been defined for use in a quantitative fashion. To determine the usefulness of the scor-
ing indices used in this study, each index was analyzed independently to determine whether the individual scores reflect the overall outcome.

INNER EAR SCORING

Initial qualitative examination of the inner ear damage, performed by 2 examiners (K.S.P. and C.G.W.), suggested that there was a relationship between the extent of damage and the concentration of AZ given. Therefore, a means was devised to estimate the damage quantitatively for inner hair cells (IHCs) and outer hair cells (OHCs) from base to apex. The severity of damage to the organs of Corti was quantified in all the AZ- and saline-treated ears, plus 5 randomly selected untreated controls. For quantification of the inner ear damage, the length of the organ of Corti was measured using a camera lucida, then the organ of Corti was separated into 10 segments of equal length from base to apex, and the tissue was analyzed in a blinded manner by 1 examiner (K.S.P.) for severity of damage of IHCs and OHCs using a scoring system with values representing specific degrees of damage (Table 2). All 3 rows of OHCs were scored together, with a score of 1 indicating similar to normal (≥90% of the hair cells present, with a near-normal appearance) and a score of 5 indicating severely damaged (no hair cells remaining in most [≥75%] of the segment). IHCs were scored separately from the OHCs, with a score of 1 indicating similar to normal (≥90% normal hair cell population) and 5 indicating severely damaged (only a very small portion [<25%] of the hair cells appearing normal).

Statistical analysis was performed using a t test on the cumulative scores for middle ear damage and on the scores for IHC and OHC damage, summed base to apex. Linear regression was used to determine the correlation between the degree of damage in the middle ear relative to the AZ concentration plus the degree of damage in the inner ear (sum of IHC and OHC damage, base to apex) relative to AZ concentration.

RESULTS

All but 1 animal survived the duration of the study. That animal had an unrecognized preexisting gastrointestinal condition and was euthanized the day after surgery. All other animals (n=22) recovered well from surgical anesthesia. Postsurgical monitoring of all animals revealed periodic circling and mild nystagmus the day after surgery in 2 of 3 animals receiving the 3% AZ solution. The symptoms were only evident when the animals were startled, and they resolved by the next day. None of the other animals (n=20) exhibited signs of vestibular upset at any time.

Pathological analysis performed 2 weeks after AZ application revealed extensive middle ear changes for all formulations tested (Table 3). Changes were seen in the ears treated with AZ compared with control ears (Figure 1A). The changes seen included the develop-

<table>
<thead>
<tr>
<th>Concentration</th>
<th>No. of Guinea Pigs</th>
<th>Mucosal Thickening</th>
<th>Hematoma</th>
<th>Hyperemia</th>
<th>Adhesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>5.0 (1.3)</td>
<td>6.6 (1.3)</td>
<td>9.3 (0.7)</td>
<td>1.5 (0.7)</td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>5.5 (0.8)</td>
<td>5.8 (1.4)</td>
<td>9.0 (2.3)</td>
<td>1.6 (0.2)</td>
</tr>
<tr>
<td>0.5% Azithromycin</td>
<td>5</td>
<td>12.6 (2.6)c</td>
<td>11.2 (1.8)c</td>
<td>8.3 (1.3)</td>
<td>3.8 (0.5)c</td>
</tr>
<tr>
<td>1.0% Azithromycin</td>
<td>5</td>
<td>12.2 (1.6)c</td>
<td>12.6 (3.0)c</td>
<td>8.2 (1.3)</td>
<td>4.6 (0.4)c</td>
</tr>
<tr>
<td>2.0% Azithromycin</td>
<td>4</td>
<td>14.0 (0.7)c</td>
<td>12.8 (2.0)c</td>
<td>9.9 (1.5)</td>
<td>4.0 (0.4)c</td>
</tr>
<tr>
<td>3.0% Azithromycin</td>
<td>3</td>
<td>12.2 (1.9)c</td>
<td>13.3 (1.0)c</td>
<td>9.8 (1.3)</td>
<td>3.8 (1.2)c</td>
</tr>
</tbody>
</table>

a Sum of 4 measurements per ear.
b Only 1 measurement per ear.
c P<.01.

Figure 1. Temporal bones from guinea pig ears that have been dissected, medial from lateral portions, to expose the middle ear. Bar=2 mm. A, Untreated control ear showing normal mucosa over the cochlea (C) on the medial half and normal tympanic membrane (T) on the lateral half of the bone. B, Ear treated with 1% azithromycin 2 weeks before death. The mucosa has large areas of hemorrhage (arrowhead), some small areas of hyperemia on the cochlea (arrow), a large adhesion overlying the cochlea (asterisk), and mucosal thickening.
ment of thick, mucosal adhesions that occupied large portions of the middle ear space and were often attached to the otic capsule (Figure 1B). Hemorrhagic and hyperemic mucosal tissue was commonly seen, and the mucosa often looked thickened. The tympanic membrane typically showed minimal changes. The most common change seen in the saline-treated ears was hyperemia (Figure 2), but this was also seen in some untreated control ears. Adhesions were never seen in saline-treated or untreated controls. Mucosal thickening and hematomas were also rare in the control ears.

Changes seen were quantitated from photomicrographs of the middle ear. The mean (SD) cumulative scores for middle ear damage were 5.8 (1.4) for untreated control, 5.8 (2.4) for saline, 9.5 (0.0) for 0.5% AZ, 9.3 (1.9) for 1% AZ, 10.0 (1.4) for 2% AZ, and 9.8 (0.8) for 3% AZ. The scores for saline-treated ears were not significantly different from those of untreated controls (P = .77, df = 4). The scores for all AZ doses were significantly different from those of controls (0.05%: P < .001, df = 4; 1%: P = .01, df = 4; 2%: P = .005, df = 3; and 3%: P = .001, df = 2).

Cochlear dissection revealed changes within the organs of Corti after AZ administration. Degree of damage varied along the organ of Corti from the cochlear apex to base and between IHCs and OHCs (Figures 3, 4, 5, 6, and 7). Little nerve fiber loss was detected: it occurred in only 1 ear that was exposed to 3% AZ and was limited to areas of complete IHC and OHC loss. OHC changes ranged from scattered hair cell loss (Figure 3A) to areas where no normal OHCs remain (Figure 4B) to total loss of hair cells and supporting cells with only thin, squamouslike cells covering the basilar membrane. IHC changes ranged from scattered hair cell loss to areas where remnants of hair cells are intermixed with normal cells (Figure 5A) to areas of complete loss of hair cells and supporting cells (Figure 3A). There was a tendency for significant IHC damage to be present in areas of minimal damage to OHCs (Figures 3A and 5A).

The IHC damage seen in this study is uncommon compared with reports of ototoxic changes seen after administration of other antibiotics.11-13 After AZ administration, some IHCs were partially or completely extruded from the organ of Corti (Figures 3-5), with only a small portion of the cell remaining tethered at the lateral side of its cuticular plate. Free-floating or dislodged IHCs were not obvious, although the reticular lamina had depressions seen by the scanning electron microscope in positions where the IHCs had been lost. B. Scanning electron micrograph of tissue from a guinea pig that received 3% azithromycin (original magnification ×1300). Tissue was demounted from glycerol after light microscopic viewing. All OHCs are present, but IHCs have degenerated. A few partially (white arrow) or completely (black arrow) extruded IHCs are present on surface of organ of Corti.
Statistically significant inner ear changes were seen for all AZ formulations tested compared with controls. Cumulative IHC and OHC scores were as follows: untreated controls, 20.5 (0.6); saline, 21.4 (1.9); 0.5% AZ, 32.4 (4.8) ($P=0.005$, $df=4$); 1% AZ, 32.2 (6.6) ($P=0.02$, $df=4$); 2% AZ, 43.0 (6.1) ($P<0.001$, $df=3$); and 3% AZ, 53.0 (12.1) ($P=0.04$, $df=2$). The scores for saline-treated ears were not significantly different from those for control ears.

To determine whether the changes seen in the middle and inner ears were owing to AZ itself or were caused by other constituents of the AZ formulation, we examined the correlation of the extent of tissue damage with the concentration of AZ present within the formulations. The extent of middle ear damage correlated moderately with AZ concentration ($r^2=0.59$). Inner ear disease was highly correlated with the concentration of AZ administered ($r^2=0.94$).

Individual indices of middle ear damage were examined for their contribution to the overall score. Mean (SD) scores for AZ-treated ears were significantly different from those for control ears for all concentrations of AZ for mucosal thickening, hematoma, and mucosal adhesions (Table 3). Although hyperemia was slightly elevated in the 2% AZ and 3% AZ ears, it was not significantly different from the untreated or saline controls.

Administration of AZ solutions to the guinea pig middle ear caused damage to the inner and middle ear. The semiquantitative methods developed yielded results that could be statistically analyzed. The moderate correlation found between AZ concentration and the extent of middle ear damage suggests that some of the damage seen in the middle ear after application was caused by AZ, but other factors appear to be involved, such as the other constituents of the test solutions. Middle ear changes caused by the nonmedicinal ingredients of otic agents have been reported.

The strong correlation of AZ concentration and inner ear damage suggests that AZ applied to the guinea pig's middle ear can cross into the inner ear and is the primary agent responsible for the ototoxicity.

The pattern of damage seen in this study, in which IHC damage is worse than OHC damage after antibiotic administration, is an unusual finding. The ototoxicity seen after administration of other antibiotics is typically greater for the OHCs or the effects for IHCs and OHCs are similar.

In addition, the observation that dying IHCs tend

Figure 4. Light microscopic images of osmium-stained, whole mount preparations from the basal region of the organ of Corti. Bars = 10 µm. A, Saline control with V- or U-shaped stereocilia bundles on the 3 rows of outer hair cells (OHCs). Normal stereocilia are also seen in the inner hair cell (IHC) region, along with a clearly visible line of IHC nuclei (original magnification $\times 1350$). B, Specimen from a guinea pig ear given 3% azithromycin showing complete OHC loss with phalangeal scarring. The only IHCs remaining have been extruded onto the endolymphatic surface (arrows) (original magnification $\times 1350$).

Figure 5. High-magnification images of the inner hair cell (IHC) region from 2 guinea pig ears treated with 3% azithromycin. A, Light microscopic image of an osmium-stained, whole mount preparation showing an extruded IHC (slanting arrow). To the left, stereocilia (vertical arrow) of a single remaining IHC are seen. Note nuclei of first-row outer hair cells (OHCs), all of which are intact (original magnification $\times 2100$). Bar = 5 µm. B, Scanning electron microscopic image of an extruded IHC on the surface of the organ of Corti. The cell remains tethered to the reticular lamina at the lateral side of the cuticular plate. Depressions can be seen on either side of this cell where the IHCs have been lost (original magnification $\times 5500$). Bar = 2 µm.
to be extruded onto the endolymphatic surface has only rarely been reported in the literature on disease of the organ of Corti. Also, in this study, the supporting cells that surround the IHCs were often found to have disappeared in areas of extensive IHC loss. This type of damage is unexpected in view of findings from previous reports on systemically administered AZ, which suggest the target tissues for AZ ototoxicity are either the stria vascularis or the OHCs. Strial changes were not obvious in our study, although the methods used were not optimal for assessment of strial disease. It is also possible that the route of administration affects the drug target. Further study is needed to better determine the nature of cell death in the organ of Corti and possible morphologic effects on the stria vascularis after ototopical AZ administration.

Because of the development of antibiotic resistance in pathogens, there is a continuing need for discovery of effective, new antibiotics for treatment of infections. Macrolide antibiotics have been shown to be effective for treatment of otitis media. Their ability to interfere with quorum sensing in biofilms suggests that they may be effective in treating the more difficult cases of otitis media where biofilms are present. Although the present study was performed using single applications on the guinea pig model, the results suggest that AZ could be ototoxic in humans if topically applied to the ear. Therefore, AZ may not be appropriate for inclusion in formulations intended for ototopical use. In addition, there have been occasional cases of temporary and permanent hearing losses after systemic AZ treatment. That fact, along with the results of the present study showing ototoxicity of AZ occurring in a concentration-dependent manner, suggest that AZ can be ototoxic if it enters the inner ear in sufficient concentrations. Therefore, caution should be taken when administering AZ systemically.

Macrolides are an accepted and often preferred therapy for chronic rhinosinusitis. Their beneficial effect is a consequence not only of their bacteriologic and bacteriostatic effect but also because they down-regulate proinflammatory mediators. They are often used in lower dosages (250 mg/d) for longer periods of time because of the immunomodulation effect. To date,
no ototoxic effects have been reported in association with this treatment regimen. However, given that oto-
toxicity has occasionally occurred in patients receiving
systemic AZ and the potential for some antibiotics to
accumulate in perilymph over time, it may be advisable
to audiometrically monitor patients who are receiving
long-term therapy.

Submitted for Publication: April 17, 2009; final revi-
sion received December 22, 2009; accepted January 12,
2010.

Correspondence: Karen S. Pawlowski, PhD, Depart-
ment of Otolaryngology–Head and Neck Surgery, The
University of Texas Southwestern Medical Center at Dal-
las, 5323 Harry Hines Blvd, Dallas, TX 75390-9035 (Karen
Pawlowski@utsouthwestern.edu).

Author Contributions: Dr Pawlowski had full access
to all the data in the study and takes responsibility for
the integrity of the data and the accuracy of the data
analysis. Study concept and design: Pawlowski, Si,
Wright, Hosseini, and Roland. Acquisition of data:
Pawlowski and Koulich. Analysis and interpretation of
data: Pawlowski and Koulich. Drafting of the manu-
script: Pawlowski. Critical revision of the manuscript for
important intellectual content: Pawlowski, Si, Wright,
Koulich, Hosseini, and Roland. Obtained funding: Si,
Wright, Hosseini, and Roland. Administrative, tech-
ical, and material support: Si, Wright, Koulich, and Hos-
seini. Study supervision: Pawlowski.

Financial Disclosure: Dr Pawlowski has applied for a
patent entitled “Micro- and non-patterned surface fea-
tures to reduce implant fouling and regulate wound
healing” (patent application 20090093879). Although
Drs Hosseini and Si are employees of the sponsor of
the study, InSite Vision Inc, they do not have any
financial interest to disclose because InSite Vision Inc
has decided not to pursue further development of an
otopically applied preparation of azithromycin, as
mentioned in this article. Dr Roland has worked as a
consultant or received honoraria from Alcon Laborato-
ries, MED-EL Corporation, Advanced Bionics LLC, and
Cochlear Corporation and has worked as a
speaker and received honoraria from GlaxoSmithKline
and Alcon Laboratories.

Funding/Support: This work was funded in part by In-
Site Vision Inc.

Previous Presentation: This study was presented at the
32nd Annual Midwinter Meeting of the Association for
Research in Otolaryngology; February 18, 2009; Balti-
more, Maryland.

Additional Contributions: Kipton Sheek, AAAS, and
Paula Timmons, HT, LAT, provided assistance in per-
foming the animal experiments. Christopher Gilpin, PhD,
Laurie Mueller, B5, and Tom Januszewski, MS, in The
University of Texas Southwestern Molecular and Cellu-
lar Imaging Facilities, provided assistance with scan-
nning electron microscopy work.

REFERENCES

1. Post JC, Hiller NL, Nistico L, Stoodley P, Ehrlich GD. The role of biofilms in oto-
2. Block SL, Cifaldi M, Gu Y, Paris MM. A comparison of 5 days of therapy with
cefdinir or azithromycin in children with acute otitis media: a multicenter, pro-
3. Guven M, Bulut Y, Sezer T, Aladag I, Eyibilien A, Etikan I. Bacterial etiology of
acute otitis media and clinical efficacy of amoxicillin-clavulanate versus
5. Girard AE, Girard D, English AR, et al. Pharmacokinetic and in vivo studies with
azithromycin (CP-62, 993), a new macrolide with an extended half-life and ex-
cellent tissue distribution. Antimicrob Agents Chemother. 1987;31(12):1948-
1954.
7. Starnes TD, Shroud JD, Parsi MA, Appelbaum PC, Kim G. Subinhibitory con-
centrations of azithromycin decrease nontypeable Haemophilus influenzae bio-
8. Jakob T, Wright CG, Robinson K, Meyerhoff WL. Ototoxicity of topical ticarcillin
10. Wright CG, Roland PS. Middle ear effects of ototopical agents. In: Roland PS,
12. Wright CG, Meyerhoff WL, Halama AR. Ototoxicity of neomycin and polymyxin
B following middle ear application in the chinchilla and baboon. Am J Otol.
13. Secane A, Dememes D, Llorens J. Relationship between insult intensity and mode
of hair cell loss in the vestibular system of rats exposed to 3,3’-iminodipropionitrile.
clarithromycin treatment on lavage-fluid markers of inflammation in chronic