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## D-METHIONINE REDUCES TOBRAMYCIN-INDUCED OTOTOXICITY WITHOUT ANTIMICROBIAL INTERFERENCE IN ANIMAL MODELS

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### Abstract

**Background**—Tobramycin is a critical cystic fibrosis treatment however it causes ototoxicity. This study tested D-methionine protection from tobramycin-induced ototoxicity and potential antimicrobial interference.

**Methods**—Auditory brainstem responses (ABR) and outer hair cell (OHC) quantifications measured protection in guinea pigs treated with tobramycin and a range of D-methionine doses. *In vitro* antimicrobial interference studies tested inhibition and post antibiotic effect assays.

**In vivo**—antimicrobial interference studies tested normal and neutropenic *E. coli* murine survival and intraperitoneal lavage bacterial counts.

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Declaration of Interest: Dr. Kathleen Campbell is the sole inventor on the patents for D-methionine as a protective agent. All of her patents are owned by her employer, Southern Illinois University School of Medicine. All other authors do not have personal relationships with organizations that could potentially be perceived as influencing the described research. All authors have read the journal's disclosure policy of potential conflicts of interest.

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**Results**—D-methionine conferred significant ABR threshold shift reductions. OHC protection was less robust but significant at 20 kHz in the 420 mg/kg/day group.

**In vitro**—studies did not detect D-methionine-induced antimicrobial interference. *In vivo* studies did not detect D-methionine-induced interference in normal or neutropenic mice.

**Conclusions**—D-methionine protects from tobramycin-induced ototoxicity without antimicrobial interference. The study results suggest D-met as a potential otoprotectant from clinical tobramycin use in cystic fibrosis patients.

## Keywords

D-methionine; aminoglycoside; otoprotection; ototoxicity; antimicrobial interference

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## 1. Introduction

Since first introduced in 1942, aminoglycoside antibiotics have increased our ability to treat gram-negative bacterial infections that are not responsive to penicillin or other conventional antibiotics (Edson and Terrell 1999). However, aminoglycoside clinical use in the United States is limited by toxic side effects that include cochleo-, vestibulo-, and/or nephrotoxicities. Preferential damage to tissue types varies by the specific aminoglycoside employed (Begg et al. 1995; Schacht et al. 2012; Francis et al. 2013). Nonetheless, because of their low cost, high efficacy, and low incidence of resistance, aminoglycoside antibiotics are commonly used in many parts of the world today including South Africa and the United States (Forge et al. 2000; Bardien et al. 2009; Harris et al. 2012; Ababneh et al. 2012). Aminoglycosides have been commonly prescribed for sepsis, meningitis, complicated urinary tract and respiratory infections because they were highly effective against gram-negative infections (McCracken 1986).

The prevalence of aminoglycoside-induced ototoxicity has been documented to be high in populations where they are monitored for possible hearing loss. Studies have reported an incidence range of 6–41% (Brummet and Fox 1989; Matz 1993; Muhleran et al. 2001). This incidence does not appear to be decreasing. Fausti et al. (1999) monitored 370 patients receiving aminoglycoside antibiotics in the Veteran's Administration system and found a 33% occurrence of aminoglycoside-induced ototoxicity. The incidence is higher in developing countries because audiologic monitoring for early ototoxicity detection is less common, dosing is less controlled than in the United States, and antibiotic options may be limited (Forge et al. 2000).

Aminoglycoside antibiotics exhibit *in vitro* activity against a variety of clinically significant gram-negative bacilli such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp., and *Pseudomonas* spp. as well as gram positive *Staphylococcus aureus* and some streptococci. As with other antibiotic classes, significant differences in the spectrum exist among the various aminoglycosides (Edson and Terrell 1999).

Each aminoglycoside induces different overall and organ-specific side effects; with particular aminoglycoside vestibulo- and/or cochleotoxicities restricting clinical use (Lodhi

et al. 1980; Aran 1995; Lima da Costa et al. 1998). Tobramycin generally causes equal cochlear and vestibular damage (Kahlmeter and Dahlager 1984; Matz 1986; Seligmann et al. 1996). Tobramycin first damages cochlear outer hair cells (OHCs) from base to apex of the organ of Corti, progressing to inner hair cells with continued use (Hawkins 1976), and causes irreversible hearing damage (Tange et al. 1982; Kusunoki et al. 2004).

In general, aminoglycosides, including tobramycin, induce reactive oxygen species (ROS) production as demonstrated in the cochlea (Hirose et al. 1997; Choung et al. 2009). These ROS can damage cochlear tissues and lead to apoptosis, loss of cochlear sensory cells, and resultant hearing loss. ROS production may be secondary to mitochondrial ribosome malfunction by causing protein mistranslation and impaired resistance to oxidative stress in the cochlea (Matt et al. 2012).

Several otoprotective agents have been proposed to prevent aminoglycoside-induced hearing loss with therapeutic targets at various steps of the proposed ototoxic pathway. However, none of the otoprotectants under development are currently FDA-approved for this purpose. Effective antioxidants, such as D-methionine (D-met), may be able to mitigate aminoglycoside-induced oxidative stress, prevent hair cell death, and reduce or alleviate subsequent hearing loss. If D-met reduces the toxicity profile of any, and hopefully all, aminoglycosides without compromising antimicrobial efficacy, a wider range of infections could be safely treated without the risk of inducing hearing loss.

D-met has effectively protected against gentamicin- and amikacin-induced ototoxicity (Sha and Schacht 2000; Campbell et al. 2007) by a presumptive free radical-detoxifying mechanism, prevention of hair cell death in the organ of Corti, and indirect cochlear mitochondrial glutathione level increases (Campbell et al. 2007; Xie et al. 2011) and may also advantageously be delivered orally (Campbell et al. 2007). This translational study tested the antioxidant D-met as an otoprotective agent against tobramycin ototoxicity and tested whether D-met administration interferes with *in vitro* or *in vivo* aminoglycoside antimicrobial efficacy.

## 2. Materials and methods

### 2.1. D-methionine otoprotection studies

**2.1.1. Subjects**—Six groups of 10 male albino guinea pigs (250–350g; approximately 5–7 weeks old) were treated with daily injections of tobramycin sulfate (100 mg/kg/day subcutaneously) for 21 days. Group 1 served as a control and received tobramycin and an equivalent-volume saline intraperitoneal injection twice daily for 21 days. Groups 2–6 received subcutaneous tobramycin and two equal intraperitoneal D-met doses totaling 240, 300, 360, 420 or 480 mg/kg each day. The D-met or saline control doses were delivered 15 minutes before and 7 hours after the daily tobramycin injection. Campbell et al. (2003) demonstrated no functional or histological change of the inner ear in animals treated only with D-met. Thus, a D-met-only-treated group was not included in this study.

All animals were maintained on a regular diet and given free access to water. After arriving at the laboratory animal facilities, animals were allowed to acclimate one week before

experiments began. Animal weight was recorded prior to each ABR assessment and once per week during drug administration. All animal protocols were reviewed and approved by Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee.

**2.1.2. Treatments**—D-methionine powder (99+% pharmaceutical grade, Acros Organics; St. Louis, Mo) was diluted into sterile normal saline at 30 mg/mL.

A 40 mg/mL tobramycin injectable solution was purchased from APP Pharmaceuticals (Schaumburg, IL).

Animals were fully anesthetized throughout ABR procedures and at sacrifice with 86.21 mg/kg ketamine (Fort Dodge; Madison, NJ) and 2.76 mg/kg xylazine (Lloyd Laboratories; Shenandoah, IA), which was supplemented as needed with half doses.

**2.1.3. Electrophysiology**—All subjects underwent auditory brainstem response (ABR) testing prior to treatment and 2, 4, and 6 weeks after initiation of drug administration. ABR monitoring used an Intelligent Hearing System evoked potential unit in a double-walled IAC sound booth. ABR thresholds were measured in both ears in response to tone-bursts with 1 ms rise/fall and 0 ms plateau gated by a Blackman envelope and centered at the frequencies of 4, 8, 14, and 20 kHz presented at 30/s. Threshold was analyzed by readers blinded to condition and defined as the lowest intensity capable of eliciting a replicable, visually detectable response at the fourth ABR waveform. A total of 512 sweeps in replicate constituted each average, and the recording epoch was 15 ms following stimulus onset.

**2.1.4. Histology**—Anesthetized subjects were sacrificed by decapitation after the final ABR measurement was obtained. Cochleae were harvested for outer hair cell counts. Round window and stapes were removed and the round and oval window was punctured, a hole was hand-drilled into the first turn of the otic capsule with a sharpened pick and the perilymphatic space was perfused with 2.5% glutaraldehyde at 4°C in 0.1M Cacodylate (Cac) buffer (pH 7.4) within 5 minutes of sacrifice; allowing the fluid to exit through the opened round window.

After perfusion fixation, the cochleae were immersed in glutaraldehyde and stored at 4°C overnight. After overnight fixation, the cochleae were rinsed in 0.1 M Cac buffer and gently perfusion-rinsed with Cac through the perilymphatic spaces 3 times. After rinsing, the cochleae were perfusion-fixed with 4°C OsO<sub>4</sub> in Cac buffer under a fume hood. Fixation continued by immersion and rotation in the same fixative for 15 minutes. The cochleae were then perfusion-rinsed three times with Cac and prepared for dissection.

Under the dissecting microscope, each cochlear bony capsule was carefully removed. The tissue was then serially dehydrated in 30%, 50%, 75%, 85%, 2 X 95%, and 3 X 100% EtOH. Each specimen was dried using hexamethyldisilazane (HMDS) and placed on a stub for sputter coating with 13 nm platinum. The tissue was viewed through a Hitachi S3000N Variable Pressure Scanning Electron Microscope at 3,000X–4,000X magnification.

**2.1.5. Outer and inner hair cell (OHC and IHC) Quantification**—OHC and IHC semiquantitative analyses were performed according to previously documented and published sampling techniques (Campbell et al. 1996; 2007, 2011; Claussen 2013). Briefly, a representative sample of 33 OHCs, or 11/row, and 11 IHCs were examined and counted for each frequency region (4, 8, 14, and 20 kHz) based off of previously published and documented frequency mapping (Eldridge et al. 1981) and the percentages of intact OHCs and IHCs were calculated. One cochlea per animal was assessed.

**2.1.6. BUN/Creatinine Analysis**—Serum samples were collected from anesthetized animals by a cardiac heart stick prior to sacrifice. Blood samples were centrifuged and serum collected for blood urea nitrogen (BUN) and creatinine analysis to identify potential permanent nephrotoxicity. BUN and creatinine levels were analyzed using an IDEX Vetest chemistry analyzer (IDEX Corporation).

## 2.2. D-methionine/tobramycin *in vitro* antimicrobial efficacy studies

**2.2.1. Isolates**—Five isolates each of the following bacteria: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) were obtained from St. John's Hospital in Springfield, IL. Isolates were subcultured twice on nutrient agar or on selective medium (e.g. MacConkey's agar for *E. coli* and Cetrimide agar for *P. aeruginosa*) for purification. Pure colonies were isolated and subcultured on nutrient agar. Bacteria were identified with API strips and Chromagar media. Fresh isolates were used to inoculate one milliliter skim milk medium and stored at  $-80^{\circ}\text{C}$ . American Type Culture Collection (ATCC) *E. coli* 25922 (clinical isolate from FDA-1946), *S. aureus* 29213 (clinical isolate from Micro-Media Systems, Inc.) and *P. aeruginosa* 27853 (blood culture isolate from AA Medeiros) were used to determine minimal inhibitory concentrations (MIC).

**2.2.2. Drugs**—Tobramycin for *in vitro* studies and D-met for both *in vitro* and *in vivo* studies were obtained from Sigma Aldrich (St. Louis, MO). Injectable forms of aminoglycosides and cyclophosphamide were obtained for *in vivo* studies. Tobramycin sulfate and cyclophosphamide were obtained from Henry-Schein (Melville, NY). A ketamine (20.8 mg/kg) and xylazine (484  $\mu\text{g}/\text{kg}$ ) cocktail was used for anesthesia. Ketamine was obtained from Fort Dodge (Madison, NJ). Xylazine was purchased from Lloyd Laboratories (Shenandoah, IA).

**2.2.3. Mean Inhibitory Concentration (MIC) Determinations**—The MIC is the lowest antimicrobial concentration which inhibits microorganismal growth. Testing for MIC can provide important information about the susceptibility of organisms to antimicrobial drugs. Aminoglycoside and D-met MICs were determined by both broth dilutions and Bioscreen C analysis for *E. coli*, *P. aeruginosa*, and *S. aureus*. Antimicrobial susceptibility tests of each aminoglycoside and D-met were determined using the broth microdilution technique as described by the Clinical and Laboratory Standards Institute (M7-A6). Antimicrobial and D-met stock solutions were diluted into Cation Adjusted Mueller Hinton Broth (CAMHB), prepared at the highest tested concentration, and stored at  $-20^{\circ}\text{C}$ . Tested serial dilutions ranged from 0.25–128  $\mu\text{g}/\text{ml}$  for aminoglycosides and 125–6,400  $\mu\text{g}/\text{ml}$  for

D-met. Five microliters of inoculum ( $1 \times 10^7$  CFU/ml) in the same medium was added to each well of the prepared plates, resulting in a final inoculum of  $5 \times 10^5$  CFU/ml. Each plate was incubated at 37°C for 18–24 hours. Following incubation, the plates were scored visually, and the MIC for each drug was determined.

#### **2.2.4. Tobramycin and D-methionine combinations against bacteria in vitro**

—Briefly, 100  $\mu$ l of a twofold serially-diluted tobramycin stock, diluted in CAMHB, was delivered to the first column of wells in a 96 well plate at twice the final concentration. D-met at twice the final concentrations (25–6,400  $\mu$ g/ml) in CAMHB was delivered to plate rows 1 to 8 (checkerboard dilution method) (Odds 2003; Lewis et al. 2002; Rand et al. 1993). Five microliters of inoculum ( $1 \times 10^7$  CFU/ml) in CAMHB was added to each well, resulting in a final inoculum size of  $5 \times 10^5$  CFU/ml. Plates were incubated at 37°C for 18–24 hours. Following incubation, the plates were scored visually. Checkerboard analyses were duplicated for each drug combination. Drug interaction was determined by calculation of the Fractional Inhibitory Concentration ( $\Sigma$ FIC) (Odds 2003). The interaction was defined as: Synergistic (FIC index  $\leq 0.5$ ); Indifferent (FIC  $> 0.5$  and  $\leq 4$ ); and antagonistic (FIC  $> 4$ ) (Odds 2003). Drug A referred to the aminoglycoside and Drug B referred to D-met.

**2.2.5. Time Course Interference Assay**—The Bioscreen C Automated Microbiology Growth Curve Analysis System (Growth Curves USA, Piscataway, NJ) directly measures microorganism growth with turbidity over time by generating an optical density (O.D.) curve (Medina et al. 2012; Cooper et al. 2011). Tobramycin MIC values against each isolate were established using a Bioscreen C prior to the time course interference assay. Tobramycin killing efficacy was tested in the presence of D-methionine (D-met) using the Bioscreen C. Tobramycin at 4x MIC were combined with serial dilution D-met concentrations (6,400  $\mu$ g/ml–100  $\mu$ g/ml) at twice the desired concentration and plated into a 100 well Bioscreen C honeycomb plate. Each combination was inoculated with  $5 \times 10^5$  CFU/ml. Samples were diluted 5 fold and plated onto agar plates for quantitation. The Bioscreen C software was programmed to take a turbidometric measurement every 15 minutes at 580nm for 24 hours. The log phase measurements were completed after 12 hours. Time course interference assays were duplicated for each tobramycin/isolate combination. Results were exported to Microsoft Excel for graph production and analysis.

**2.2.6. Post Antibiotic Effect (PAE) Studies**—Post-antibiotic effect (PAE) is defined as the length of time bacterial growth is suppressed following brief exposure to an antibiotic. This pharmacodynamic parameter has long been studied as part of preclinical evaluations to determine dosing intervals. Aminoglycosides exhibit rapid concentration-dependent killing action; therefore, PAE is of particular interest due to short exposure time associated with persistent bacterial growth suppression (Craig 1991). Higher aminoglycoside dosages give greater post-antibiotic effects up to a certain maximal response. *In vivo*, the aminoglycosides post-antibiotic effect is lengthened by the synergistic effect of host leukocyte activity, with possible enhanced leukocyte phagocytic capabilities, following aminoglycoside exposure (Gonzalez and Spencer 1998). PAE was determined for the MIC, 2X MIC, 5X MIC, and 10X MIC of aminoglycosides for each isolate. Aminoglycosides at twice the desired concentration were combined with serial dilution D-met concentrations (6,400  $\mu$ g/ml to 400



µg/ml) at 2x the desired concentration and placed into 125 mL Erlenmeyer flasks, to determine the effect of D-met on aminoglycoside PAE. Each flask was inoculated with the tested isolate at  $5 \times 10^5$  CFU/ml and placed in a 37°C shaker for 2 hours. Each sample inoculum was diluted 5 fold and plated onto agar plates for quantitation. Flask contents were then transferred to centrifuge tubes, centrifuged at 500 x g, and washed three times with normal saline. Following the washing process, the remaining pellets were resuspended in CAMHB. Samples were then plated into a 100 well Bioscreen C honeycomb plate. An inoculum sample was diluted 5 fold and plated onto agar plates for quantitation. The Bioscreen C software was programmed to take a turbidometric measurement every 15 minutes at 580nm for 24 hours. PAE determinations were completed after six hours. Results were exported to Microsoft Excel for graph production and analysis.

### 2.3. D-methionine/tobramycin *in vivo* antimicrobial efficacy studies

**2.3.1. Animals**—White, male, specific pathogen free, Swiss-Webster mice weighing 18 to 20g (approx. 6–8 weeks old) were used in all *in vivo* antimicrobial studies. All animal care and use was approved by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee and were performed under the supervision of the Southern Illinois University School of Medicine Division of Laboratory Animal Medicine.

We tested the possible D-met effect on aminoglycoside therapeutic efficacies in a lethal *E. coli*-infected murine model. Since antagonism may be clinically significant only in the host with diminished phagocytic function (Sande and Overton 1973), these studies were performed in both normal and neutropenic mice. This determination used a two-step process. Initially LD<sub>50</sub> was determined in both healthy and neutropenic mice. These mice were used in the second phase of the study. The second phase determined if D-met interferes with tobramycin's antimicrobial action *in vivo*. Result evaluations measured the length of animal survival as well as peritoneal cavity bacterial counts at the time of sacrifice.

**2.3.2. Induction of Neutropenia**—Cyclophosphamide, diluted in sterile saline immediately before use, was administered intraperitoneally (ip) in three equally divided doses (Days 0, 1, and 3) totaling 350 mg/kg. Animals were anesthetized using a ketamine (20.8 mg/kg) and xylazine (484 µg/kg) cocktail for sub-mandibular bleeding. Manual leukocyte and differential counts were performed on collected blood samples on days 2 through 4. Mice with neutrophil counts <500 neutrophils/µl were considered neutropenic (Han and Cutler 1997).

**2.3.3. Inoculum preparation**—*E. coli* was cultured overnight in CAMHB at 37°C. Cultures were diluted 1:10 and rotated on an orbital shaker for 1 hour in a 37°C water bath to reach log-phase growth. The suspension was standardized at  $1 \times 10^8$  colony-forming units (CFU)/ml in CAMHB using a spectrophotometer and subsequently diluted in CAMHB to obtain the desired concentration. The final inoculum quantitation was established using bacterial plate counts.

**2.3.4. Determination of Infective lethal dose (LD<sub>50</sub>)**—Healthy and neutropenic mouse *E. coli* (ATCC 25922) LD<sub>50</sub> levels were established. Neutropenic mice were used to model diminished phagocytic function in humans (Sande and Overton 1973). The LD<sub>50</sub> determined an optimal challenge dose for our model prior to proceeding with the antimicrobial interference assays (Braun et al. 1981; Traub 1982; Lobry et al. 1992; Gottfredsson et al. 1995; Dominguez et al. 2001; Li and Tang 2004; Pliego-Castaneda et al. 2005).

By definition the LD<sub>50</sub> establishes the number of microorganisms of a particular species that will kill 50% of a given animal population. We needed to first establish the LD<sub>50</sub> in order to establish a challenge dose for our antimicrobial combination. The challenge dose was calculated such that a given concentration, based on the LD<sub>50</sub>, should kill all untreated mice. Soothill et al. (1992) established that values above the LD<sub>50</sub> were appropriate for interference studies. If the challenge inoculum is too small, an infection will not be established. The antimicrobial efficacy was determined by the protection afforded the challenged mice. No *E. coli* ATCC 25922 LD<sub>50</sub> data has previously been published in the Swiss-Webster strain of mice. ATCC was unaware of an LD<sub>50</sub> in mice using this *E. coli* strain.

Seven groups of 3 healthy mice and seven groups of 3 neutropenic mice, for a total of 42 mice, were injected ip with 0.30 ml of 7 different *E. coli* concentrations escalating from 10<sup>2</sup> to 10<sup>8</sup> CFU/ml in 10<sup>1</sup> increments. In neutropenic mice, infection was induced on the 4<sup>th</sup> day of neutropenia induction. LD<sub>50</sub> was defined as the bacterial concentration at which 2 out of 3 of the animals in any group died within 5 days following inoculation.

**2.3.5. Antimicrobial interference studies**—Normal (non-neutropenic) and neutropenic mice were injected ip with inoculum at 10X the established LD<sub>50</sub> levels or saline for control mice. Infected mice were divided into 4 treatment groups: saline; antibiotic only; D-met only; or antibiotic/D-met in combination. Normal and neutropenic mice were treated 5 times at 2, 12, 24, 36 and 48 hours after inoculum by ip injection with: saline (control group); D-met (200 mg/kg); tobramycin (15 mg/kg); or a D-met (200 mg/kg)/ tobramycin (15 mg/kg) combination. Eight hours post-inoculation, three mice from each group were euthanized and peritoneal lavage bacteria counts were quantified. The remaining five mice in each group were observed for survival 5 days following inoculation.

## 2.4 Statistical Analysis

**2.4.1. Electrophysiology**—ABR threshold shift statistical analyses employed an Analysis of Variance (ANOVA) design in which D-met dosage was listed as a between subject variable and observation intervals (time) were within subject variables (repeated measure). Frequency was also a within subject variable nested within observation intervals. The major result of interest was the group (treatment) x time interaction. The between subjects factor (grouping variable) was the dose of D-met given with six levels (one control and 5 treatments) and the within subject variables were frequency (4 levels) and date of testing (3 levels).



For the ABR measurements, the left and right ear thresholds were first compared to check for a difference between ears. No significant differences were observed; thus, data from the each left ear only was used for histologic studies to eliminate concerns of using both right and left ears as independent data points. Baseline ABR thresholds were analyzed separately by a two factor ANOVA with frequency as a repeated measure with Dunnett's post-hoc follow-up at  $p = 0.05$  and  $p = 0.01$  to compare D-met-treated groups to control groups. Tukey's post-hoc follow-up also measured pairwise comparisons between D-met-treated groups at  $p = 0.05$  and  $p = 0.01$ .

**2.4.2. OHC quantification**—OHC percentages were statistically analyzed by a two-way ANOVA with repeated measures (frequency) with Dunnett's post-hoc follow-up at  $p = 0.05$ . The between subjects factor as group with six groups (one control and five treatment groups) and within subjects factor as frequency region with four levels (4, 8, 14 and 20 kHz regions).

**2.4.3. Antimicrobial efficacy**—To compare different antimicrobial agent growth phase shifts *in vitro*, an optical density (OD) band ranging from 0.2 to 0.8 was used as an isolate linear growth phase filter. For every OD in that band, time was recorded to provide a mean and standard deviation for each agent. Thus, for every linear growth phase, an average time to the event comparison could be performed between the different combinations. This average time was then compared between the different combinations with an Analysis of Variance with Dunnett's post-hoc follow-up at  $p = 0.05$ .

*In vivo* antimicrobial efficacy statistical analysis comprised Fisher's exact test for percent survival studies and an independent t-test for bacterial quantification. *In vivo* analysis compared D-met/Tobramycin combinations to mouse groups treated only with tobramycin.

Statistical significance for all tests was set at  $\alpha$ -levels  $p = 0.05$  and  $p = 0.01$ . Statistical analyses were performed with SAS 9.1 software.

### 3. Results

#### 3.1. Electrophysiology

Overall ABR analysis revealed significant group effects ( $F = 9.98$ ,  $df = 5$ ,  $p = 0.0001$ ) and no interaction between group and frequency ( $F = 1.49$ ,  $df = 15$ ,  $p = 0.1145$ ). Baseline thresholds are reported in Figure 1.

At 2 weeks, D-met ABR threshold shift protection was significant at the .01 level for the 360 mg/kg/day dosing group at 4 and 8 kHz (Figure 2). As expected, ABR threshold shifts in controls increased over time. At two weeks post-tobramycin initiation, mean control group ABR threshold shifts ranged from 6.5–13.5 dB; with the greatest threshold shifts at 4 and 8 kHz. Two week threshold shift for D-met dosing groups 240, 300 and 480 mg/kg/day tended to be less than controls but did not reach significance; possibly because of the minimal threshold shifts measured in the 2 week control group. D-met doses of 360 mg/kg/day resulted in threshold shift less than 5 dB at all frequencies, and 420 mg/kg/day doses resulted in threshold shift less than 5 dB at 8 and 14 kHz (Figure 2) at two weeks. The 360

mg/kg/day D-met group measured significant ABR threshold shift reductions compared to the 240 mg/kg/day at 4 and 20 kHz at the 0.05 level. The 360 mg/kg/day D-met group also measured significant ABR threshold shift reductions compared to the 480 mg/kg/day group at 8 kHz at the 0.05 level (Figure 2).

D-met protection was more evident at four weeks because threshold shift was greater in the controls at that time point allowing greater contrast to protected groups. ABR threshold protection was significant at the .01 level for all frequencies for 360 and 420 mg/kg/day D-met dose groups and for the 480 mg/kg/day D-met group at 4 kHz. Additionally D-met protection occurred at the .05 level for 4 kHz at the 240 mg/kg/day dosing epoch and for 8 kHz at the 480 mg/kg/day D-met dose. At four weeks post-tobramycin initiation, mean control group ABR threshold shifts ranged from 28–35 dB; with the greatest shifts at 14 and 20 kHz. The most effective D-met doses of 360 and 420 mg/kg/day both resulted in ABR threshold shift less than 10 dB at 4 and 8 kHz (Figure 2) at four weeks. The 360 and 420 mg/kg/day D-met-treated group measured significant ABR threshold shift reductions compared to the 240 mg/kg/day group at 4, 14, and 20 kHz at the 0.05 level (Figure 2).

The greatest D-met protection of ABR thresholds related to controls was observed at 6 weeks. Mean control group ABR threshold shifts assessed six weeks post-tobramycin initiation (Figure 2) ranged from 34–36.5 dB. D-met doses of 360 and 420 mg/kg/day both resulted in threshold shift less than 10 dB at 4 and 8 kHz, and additionally at 14 kHz in the 360 mg/kg/day group. Threshold shift at 6-week ABR analyses of all D-met dosing groups averaged 11.5, 14, 16.9 and 20.2 dB for the tested frequencies of 4, 8, 14, and 20 kHz, respectively. Significant otoprotection occurred at the .01 level for all frequencies at the 360 and 420 mg/kg/day dosing levels. For the 300 mg/kg/day dosing level, protection was significant at 4 and 8 kHz at the .01 level and at 14 kHz at the .05 level. For the 240 mg/kg/day level, significant protection occurred at the .01 level for 4 kHz and at the .05 level for 8 kHz. For the 480 mg/kg/day D-met dosing level, significant protection occurred at the .01 level for 4 and 8 kHz but not for 14 or 20 kHz. The 360 and 420 mg/kg/day D-met-treated group measured significant ABR threshold shift reductions compared to the 240 mg/kg/day group at 4 and 14 20 kHz at the 0.05 level (Figure 2). The 360 mg/kg/day D-met-treated group also measured significant ABR threshold shift reductions compared to the 240 mg/kg/day group at 4 and 14 20 kHz at the 0.01 level (Figure 2).

### 3.2. Histology

Overall OHC counts revealed significant group effect ( $F = 2.53$ ,  $df = 5$ ,  $p = 0.0394$ ) and no interaction between group and frequency ( $F = 1.42$ ,  $df = 15$ ,  $p = 0.1430$ ) (Figure 3). Dunnett's follow-up revealed a significant difference between the 420 mg/kg/day D-met dosing group at 20 kHz and treated control at  $p = 0.05$ . The mean percentage of remaining OHC present in all tested frequencies for the tobramycin-treated control group ranged from 48–77%, in comparison to groups treated with tobramycin and 360 and 420 mg/kg/day D-met; which ranged from 69–87% and 71–88%, respectively. Thus, D-met-treated animals demonstrated increased remaining OHC percentages compared to controls (Figures 3, 4).

### 3.3. BUN/Creatinine

No differences in BUN and creatinine occurred between groups. BUN concentrations ranged from 18.1–20.8 across all groups. Creatinine concentrations ranged from 0.33–0.37 across all groups. Even in the tobramycin-treated controls, BUN and creatinine values were within normal limits suggesting that this tobramycin ototoxicity animal model does not induce nephrotoxicity. D-met did not influence BUN or creatinine measurements; suggesting that D-met also does not induce nephrotoxicity. Thus, we could not test for D-met protection from nephrotoxicity because the tobramycin-treated controls did not exhibit nephrotoxicity.

### 3.4. Minimal Inhibitory Concentration (MIC)

The MICs against *E. coli* isolates ranged from: 1–64 µg/ml (tobramycin); and 6,400 µg/ml (D-met). The MICs for *Staphylococcus aureus* isolates ranged from: 0.5–4 µg/ml (tobramycin); and 6,400 µg/ml (D-met). The MICs against *Pseudomonas aeruginosa* ranged from: 0.5–128 µg/ml (tobramycin); and 6,400 µg/ml (D-met). All ATCC control strains measured aminoglycoside MICs within expected ranges.

### 3.5. Mean Fractional Inhibitory Concentration Index (FIC)

FIC shows tobramycin indifference to all tested D-methionine (D-met) concentrations. At high D-met concentrations, tobramycin/D-met (6,400 µg/ml) combinations showed synergy in 42% of tested isolates. After repeated trials, one *P. aeruginosa* isolate was identified as an extreme outlier and removed from analysis because it measured non-replicable variability that was significantly greater than the average. Thus, these results do not demonstrate antagonism at high D-met concentrations for any of the isolates tested (Figure 5).

### 3.6. Time course inhibition assays

Tobramycin microbial inhibitory efficacy in combination with D-met was tested using time course microbial inhibition assays. The Bioscreen C instrument was used to establish inhibitory curves (Cooper et al. 2011). Bioscreen C-determined MIC's were coordinated with each time course inhibition to optimize the Bioscreen C system to each isolate. Growth during the logarithmic phase was used to analyze whether D-met affects the aminoglycoside inhibitory efficacy over time. The time period during the log growth phase was used to determine whether D-met had an inhibitory antimicrobial effect on tobramycin. D-met alone at 6,400 µg/ml produced growth curves which paralleled bacterial growth rates without antimicrobials, which indicates that D-met did not have an inhibitory effect on bacterial growth. All generated tobramycin/D-met combination growth curves, when compared to the media control, showed no inhibitory effects (Figure 6). Effect comparisons on tobramycin tested against typical clinical isolates indicated no D-met-induced antimicrobial interference.

### 3.7. Post Antibiotic Effect (PAE)

Experimentation was performed using the *E. coli* model system to further test for tobramycin/D-met PAE interactions. PAE studies confirmed growth inhibition assay results. No tobramycin/D-met combinations produced growth curves with a shorter lag growth phase than either the *E. coli* alone or D-met only control. Optical density (OD<sub>580</sub>) measurements after D-met and aminoglycoside removal revealed that a 4–5 hour PAE was produced in each

of the original antimicrobial combinations (Figure 7). Microbial growth resumption began approximately 5–6 hours after tobramycin removal. PAE results were not affected when *E. coli* was treated with D-met/tobramycin combinations. These results were consistent with all tobramycin MIC's tested and at all D-met concentrations. PAE studies further confirmed results found in the time course inhibition assays. Across all antibiotics, D-met and tobramycin combinations did not grow before D-met and isolate controls. Therefore, PAE experiments did not detect D-met-induced antimicrobial inhibition.

### 3.8. Antimicrobial efficacy *In vivo* studies

D-met did not significantly affect *in vivo* tobramycin efficacy in either normal or neutropenic mice challenged with 10 LD<sub>50</sub> of *E. coli* (1×10<sup>5</sup> CFU). Peritoneal lavage counts performed 8 hours post-antibiotic treatment showed no significant bacterial increases for tobramycin-/D-met- treated mice over tobramycin-treated mice. No significant bacterial increases in either normal (p = 0.110) or neutropenic (p = 0.414) mice were seen in any of the tested tobramycin treatment groups (Table 1).

D-met combined with tobramycin treatments did not significantly influence mouse survival rates for normal (p = 0.444) or neutropenic (p = 0.166) mice compared to groups treated only with tobramycin. However, D-met/tobramycin combination groups measured increased survival compared to tobramycin-alone groups that did not achieve statistical significance (Table 1).

## 4. Discussion

### 4.1. D-met confers dose-dependent protection

This study demonstrates D-met's ability to significantly decrease tobramycin-induced ototoxicity via reducing ABR permanent threshold shifts and decreasing OHC loss. To our knowledge, we are the first to report dose-dependent D-met otoprotection against tobramycin. D-met doses at 360 and 420 mg/kg/day administered 15 minutes prior to and 7 hours after tobramycin resulted in a highly significant ototoxicity reduction at all ABR-tested frequencies. Doses below 360 and above 420 mg/kg/day showed a lesser degree of protection and suggest an optimal bi-phasic therapeutic D-met dosing range exists in tobramycin combinations. While significant otoprotection at some frequencies was detected in all dosing groups, 360 and 420 mg/kg/day D-met doses showed the most robust significance across all frequencies tested. This pattern was detected histologically by OHC analysis in which the 420 mg/kg/day dosing group showed significant otoprotection at 20 kHz using a Dunnett's Test. From a descriptive standpoint, the average percent remaining OHCs is higher in the 360 and 420 dosing categories than in the controls for all frequency regions which is consistent with the same dosing levels and frequency for ABR measures. However the variability and multiple measures, while correcting for Bonferroni effect for multiple comparisons, diluted the analyses and precluded us from reaching statistical significance at all the 420 dosing level at 20 kHz. Mechanistically, some of the protection reflected in the ABRs may also have been contributed by protection of the spiral ganglion cells or another area however this hypothesis requires further investigation.

Clinical D-met dosing ranges for protection from tobramycin are not yet determined. However, clinical tobramycin dosing, ranging from 8–15 mg/kg/day for cystic fibrosis patients (Massie and Cranswick 2006; Flume et al 2009), is a fraction of the high dose (100 mg/kg/day) used to rapidly induce ototoxicity in the animal model. Although a clinical D-met dose is not yet determined to protect from tobramycin, D-met has demonstrated significant protection from noise-induced hearing loss in animal models with low doses (Clifford et al. 2011). Therefore, although somewhat preliminary, the dose-dependent protection measured in the current study and D-met's previous low-dose protection from noise suggests that optimal D-met protection from clinically-prescribed tobramycin may also require only a fraction of the currently tested D-met doses. Further clinical D-met dosing for tobramycin ototoxicity is warranted.

In this study, we also measured body weight, BUN, and creatinine. Comparisons showed no significant difference between treated controls and D-met experimental groups for these measures; although the saline included with intraperitoneal injections may have eliminated any nephrotoxicities or protection by flushing the kidneys throughout the experiment. Animals may have recovered from acute nephrotoxicity 6 weeks post-treatment. However, unchanged BUN and creatinine measures suggest that D-met may not induce permanent nephrotoxicity.

#### 4.2. D-met does not interfere with tobramycin antimicrobial efficacy

Based on limited published reports, D-met does not interfere with aminoglycoside antimicrobial action (Herr et al. 2001) and does not alter serum gentamicin levels (Sha and Schacht 2000). Therefore, we postulated treatment efficacy should be retained even with D-met protection from ototoxicity. Although little information is available regarding D-met's antimicrobial activity, some evidence indicates that D-met may itself have antimicrobial activity and enhance the antimicrobial activity of other antibiotics. D-met, at high concentration, had lethal effects against bacteria when used alone due to its incorporation into the peptidoglycan layer of the cell wall (Caparros et al. 1992). This report was not supported by our data; indicating that at 6,400 µg/ml D-met concentrations, no significant logarithmic growth reduction was observed.

We studied *E. coli*, *P. aeruginosa* and *S. aureus* clinical isolates for their growth response in the presence of tobramycin, D-met, and D-met/tobramycin combinations. We further studied the effect of these combinations in an *in vivo* murine model using *E. coli* infections. These data support the observations of Herr et al. (2001) who reported that D-met does not interfere with aminoglycoside antimicrobial activities. Time course assays using tobramycin against *E. coli*, *P. aeruginosa* and *S. aureus* clinical strains indicated no D-met interference with tobramycin antimicrobial efficacy. D-met alone did not have an inhibitory effect on the microorganismal growth rate. Further, the use of D-met in combination with tobramycin had no effect on their post antibiotic effect in a murine model.

A murine model of lethal *E. coli* infection did not report significant antagonism with D-met/tobramycin combinations. Antagonism, if it exists, may be clinically significant only in a host with diminished phagocytic function (Sande and Overton 1973); thus, we performed experiments in both normal and neutropenic mice. Tobramycin *in vivo* antimicrobial activity

was not affected by D-met in either neutropenic or non-neutropenic mice. In fact, D-met combined with tobramycin therapy may have increased survival in the murine model compared to isolate only and tobramycin only controls in normal and neutropenic mice (Table 1); possibly through its incorporation into the peptidoglycan layer of the cell wall (Caparros et al. 1992). Thus, D-met administration may protect from multiple tobramycin-induced adverse drug reactions.

Another sulfhydryl antioxidant compound, N-acetyl cysteine (NAC), has also been tested to prevent aminoglycoside-induced hearing loss. However, when tested for protection from kanamycin-induced ototoxicity, NAC actually exacerbated hearing loss (Bock et al. 1983). Further, NAC demonstrated antagonistic effects against gentamicin and tobramycin antimicrobial efficacy (Parry and Neu 1977) and demonstrated increased but insignificant gram-negative bacterial incidence in clinical trials (Feldman et al. 2007). D-met provided protection from aminoglycoside-induced ototoxicity (Sha and Schacht 2000; Campbell 2003; current study) and has now demonstrated a lack of interference with aminoglycoside antimicrobial efficacy. Thus, although D-met and NAC are both antioxidant compounds, they may protect by different mechanisms.

Aminoglycoside ototoxicity, aminoglycoside bactericidal efficacy and D-met otoprotection act by different mechanisms. Aminoglycosides induce ototoxicity by inducing iNOS pathways and creating hydroxyl radicals via the Fenton Reaction, which induces lipid peroxidation and activates cell death via apoptotic and necrotic pathways (Sha and Schacht 1999). Aminoglycoside bactericidal efficacy, however, is dependent on 30s ribosomal inhibition, protein synthesis inhibition, and resultant cell death. Although D-met otoprotective mechanisms have not been fully elucidated, it is known to be a direct and indirect antioxidant and has not demonstrated interaction with 30s ribosomal inhibition. To the best of our knowledge, this study is the first *in vitro* and *in vivo* antimicrobial interference study testing D-met and aminoglycoside combinations. Our results also confirm previous studies: D-met does not alter the *in vivo* or *in vitro* aminoglycoside antimicrobial activity against either gram positive or gram negative bacteria.

#### 4.3. Clinical implications and impact

Currently, no FDA-approved agent exists for protection from tobramycin-induced ototoxicity. Our current findings suggest D-met affords highly significant ( $p = 0.01$ ) otoprotection from tobramycin and shows promise for improving quality of life outcomes for patients receiving high dose or repeated tobramycin treatments.

Tobramycin- and aminoglycoside-induced ototoxicity reports range from nearly absent to approximately 35% of cystic fibrosis patients (Mulheran et al. 2001, 2006; Piltcher et al. 2003; Cheng et al. 2009; Guy et al. 2010; Martins et al. 2010; Scheenstra et al. 2010; Al-Malky et al. 2011; Hennig et al. 2014) with an increased risk of aminoglycoside-induced hearing loss with repeated and higher cumulative doses. The majority of adolescent and adult cystic fibrosis patients are colonized with *Pseudomonas aeruginosa* (*P. aeruginosa*) (Cystic Fibrosis Foundation Registry 2011). These patients require long-term intravenous and inhaled tobramycin dosing regimens due to chronic and acute *Pseudomonas aeruginosa*



infections (Treggiari 2009; Prayle and Smyth 2010). Thus, a large cystic fibrosis patient population may benefit from D-met otoprotection.

*P. aeruginosa* is also a common cause of respiratory infections in intensive care unit and ventilator-dependent patients (American Thoracic Society 2005) that require tobramycin treatment. Many patients with these respiratory infections have multiple co-morbid health conditions that are typically more serious with advanced age (American Thoracic Society 2005). Aging is associated with decreased glutathione levels, reduced natural antioxidant defenses and cellular damage, mechanisms similar to ototoxic aminoglycosides (Lautermann et al. 1997). D-met's antioxidant characteristics may be particularly helpful in these tobramycin-treated patient populations.

Internationally, aminoglycosides are frequently utilized for infections because of low cost, infrequent drug-induced allergy, and accessibility. However, in developing countries, aminoglycosides are more likely to result in ototoxic damage secondary to widespread use without physician monitoring and, infrequent audiologic evaluation (Oghan et al. 2011). Social factors such as stress and poor nutritional status may also predispose patients to aminoglycoside ototoxicity and increase drug-induced hearing damage particularly in developing nations (Forge and Schacht 2000). Thus, D-met therapy combined with aminoglycosides may prevent ototoxicity internationally without compromising aminoglycoside antimicrobial efficacy.

#### 4.4. Future studies

This study provides evidence supporting D-met as an otoprotective therapeutic agent, particularly for an aminoglycoside with varied cochlear and vestibular toxicities, without exacerbating antimicrobial efficacy. D-met combined with tobramycin may allow for longer dosing intervals with decreased concern for hearing loss. Future human clinical trials are now needed to assess D-met's safety and efficacy with tobramycin to confirm these *in vivo* and *in vitro* studies.

The required preclinical functional measures for FDA submission are protection from auditory threshold shift which we measure with auditory brainstem response (ABR). For clinical ototoxicity monitoring, the standards are based on auditory threshold measures, both in the conventional and high frequency audiometric ranges (ASHA 1994, AAA 2009). Otoacoustic measures are not a part of the required clinical ototoxicity standards. Thus, distortion product otoacoustic emissions (DP-OAEs) and spiral ganglion cell assessment did not meet the current study's translational bench to bedside focus. However, they would be of great interest in future studies of specific OHC function, protection, and lesion sites; particularly to address the protection differences measured between auditory threshold and OHC quantification analysis.

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## Abbreviations

<b>ABR</b>	auditory brainstem response
<b>AG</b>	aminoglycosides
<b>ANOVA</b>	analysis of variance
<b>ATCC</b>	American Type Culture Collection
<b>BUN</b>	blood urea nitrogen
<b>CAMHB</b>	cation-adjusted Mueller-Hinton broth
<b>CFU</b>	colony forming unit
<b>D-met</b>	D-methionine
<b>DP-OAEs</b>	distortion product otoacoustic emissions
<b>HMDS</b>	hexamethyldisilazane
<b>IHC</b>	inner hair cell
<b>IP</b>	intrapertitoneal
<b>MIC</b>	minimum inhibitory concentration
<b>FIC</b>	fractional inhibitory concentration
<b>OHC</b>	outer hair cell
<b>PAE</b>	post-antibiotic effect
<b>ROS</b>	reactive oxygen species

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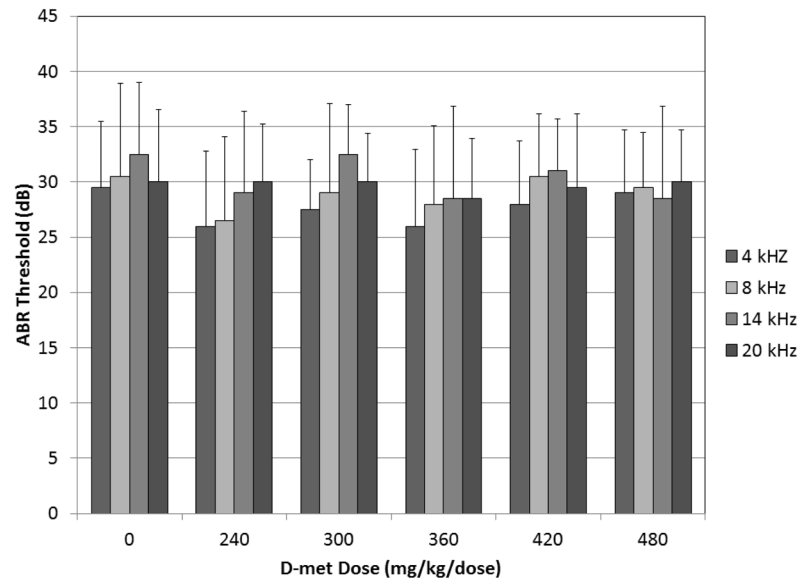


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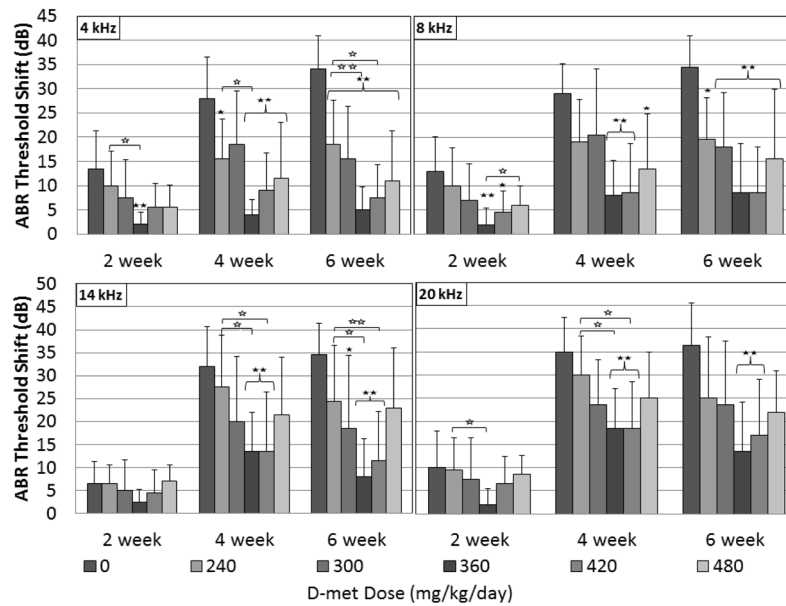
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**Highlights**

- D-met protects from tobramycin-induced ototoxicity as early as 2 weeks after administration.
- D-met protection is dose-dependent and increases over time.
- D-met does not interfere with aminoglycoside antimicrobial efficacy.
- D-met protection may help cystic fibrosis or other vulnerable patient populations.

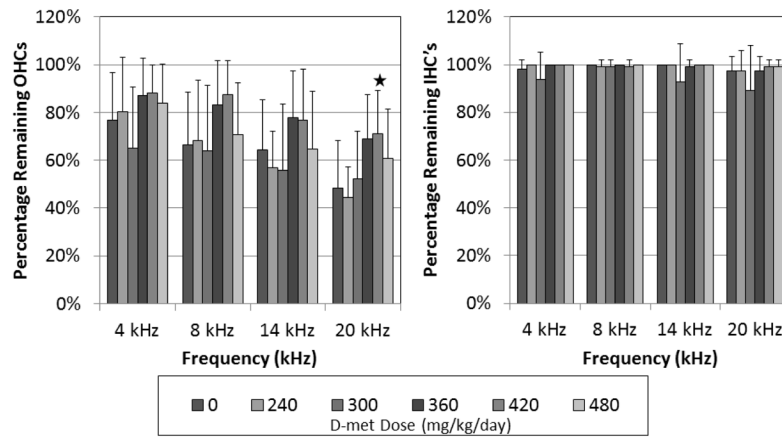


**Figure 1.** Baseline ABR thresholds for 4, 8, 14, and 20 kHz tone-burst frequencies for all tobramycin-treated control and D-met dosing groups.



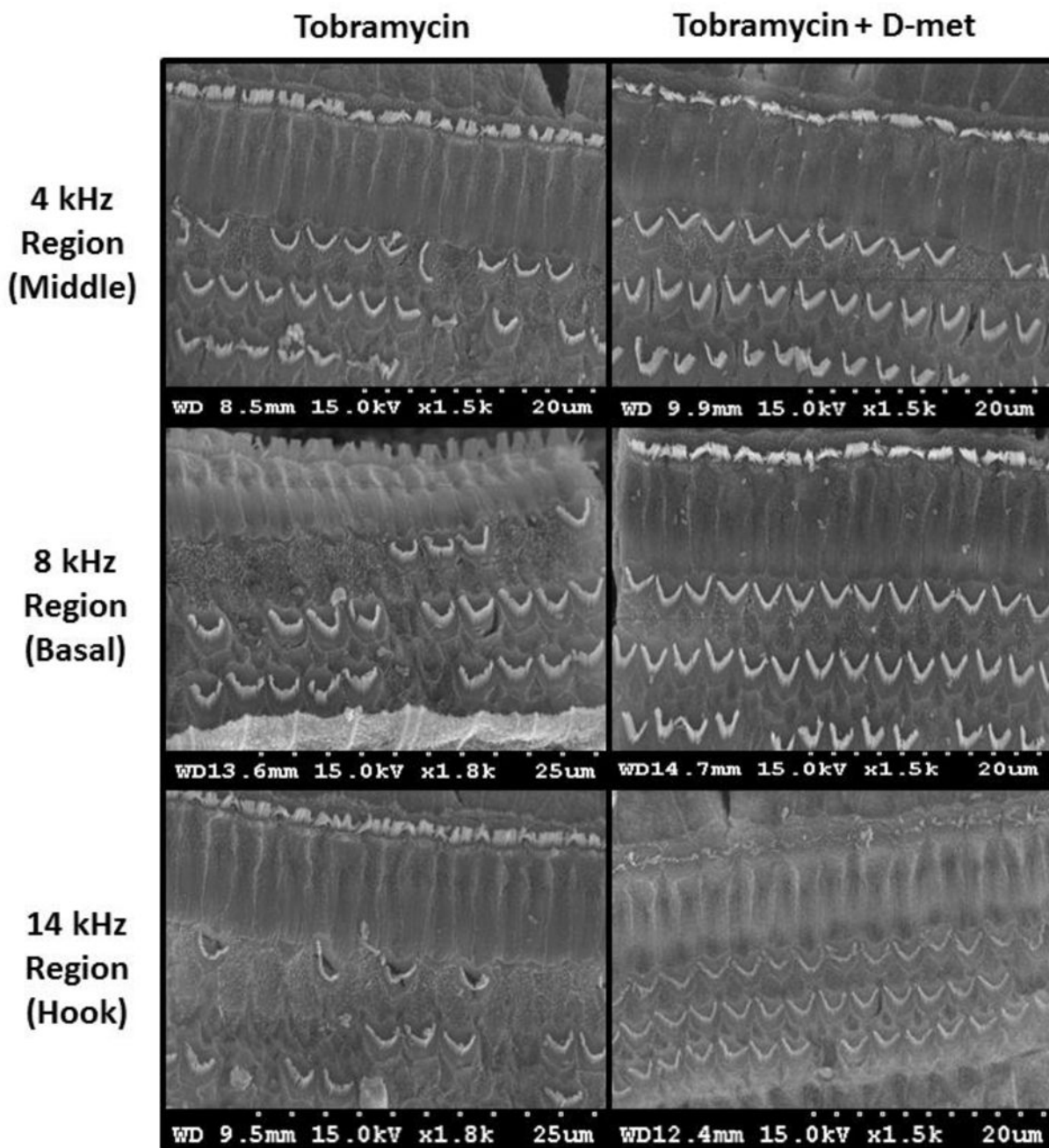
**Figure 2.**

ABR threshold shifts at 2, 4, and 6 weeks for 4, 8, 14 and 20 kHz tone-burst frequencies for all tobramycin-treated control and D-met dosing groups. Six guinea pig groups ( $n=10$ ) were treated daily with 100 mg/kg/day tobramycin sulfate for 21 days. Five experimental groups received doses of 240, 300, 360, 420 or 480 mg/kg/day D-met 15 min prior to and 7 hours after each tobramycin administration. Significant results differing from the control group at an  $\alpha$ -level of 0.05 or 0.01 as determined by a Dunnett's post-hoc test are indicated by one or two closed stars, respectively. Significant results differing from D-met-treated groups at an  $\alpha$ -level of 0.05 or 0.01 as determined by a Tukey's post-hoc test are indicated by one or two open stars, respectively. Error bars represent  $\pm 1$  SD.

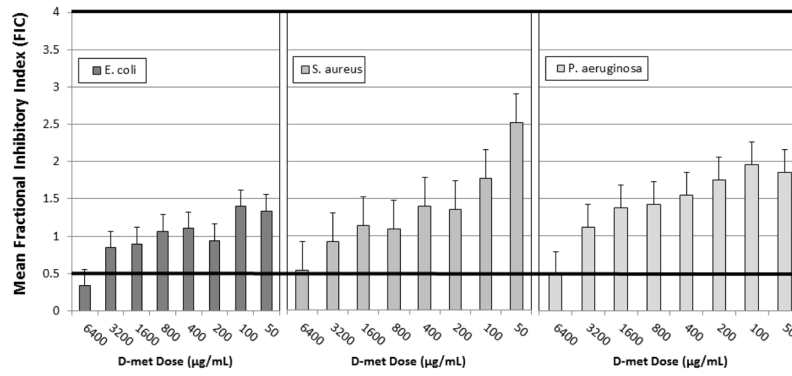


**Figure 3.** Remaining outer and inner hair cell percentages at sacrifice for treatment groups at 4, 8, 14 and 20 kHz tone-burst frequencies. Three weeks after a 21 day treatment course, cochleae were harvested and prepared for OHC viewing and analysis. Significant results differing from the control group at an  $\alpha$ -level of 0.05 using Dunnett's test are indicated with one star. Error bars represent  $\pm$  1 SD.

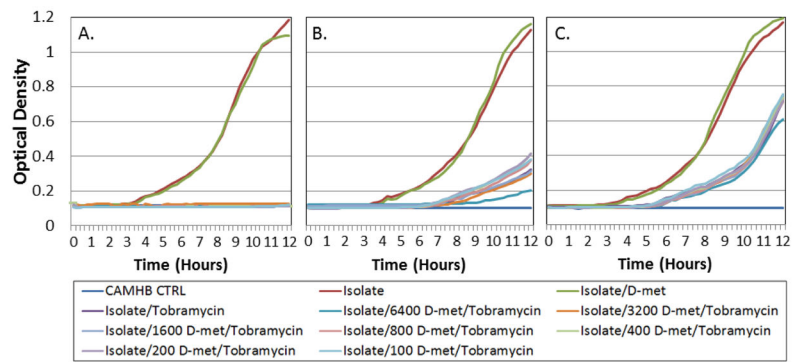




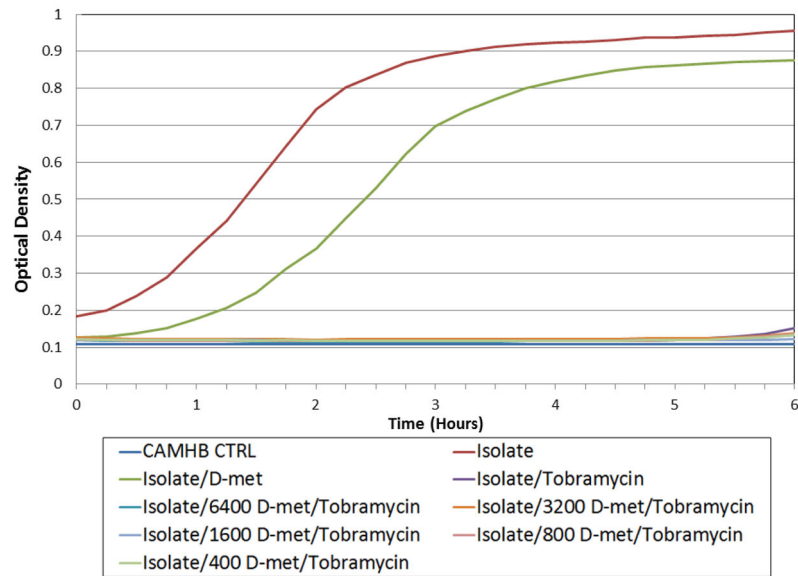
**Figure 4.** Cochlear outer hair cell SEM micrographs of animals treated with tobramycin and saline (left) or tobramycin and D-met (420 mg/kg/dose); right) in the 4 kHz (middle), 8 kHz (basal), and 14 kHz (hook) cochlear regions. Three weeks after a 21 day treatment course, cochleae were harvested and prepared for OHC viewing and analysis. D-met treatment significantly increased remaining OHC counts only in the 20 kHz region ( $p < 0.05$ ). However, increased OHC counts were observed throughout the cochlea. Scale for each image ranges from 20–25  $\mu\text{m}$ .



**Figure 5.** Mean Fractional Inhibitory Index (FIC) for tobramycin and D-met concentrations. A checkerboard assay was used to determine MIC values of tobramycin (128–0.25 µg/ml) in combination with a serial dilution of D-met (6400–100 µg/ml). FIC values were calculated using previously determined MIC results for tobramycin and D-met and *E. coli*, *S. aureus*, or *P. aeruginosa* (n = 6 for *E. coli* and *S. aureus*; n = 5 for *P. aeruginosa*).



**Figure 6.** Clinical isolate time course inhibition assays for tobramycin/D-met combinations. Graphs show optical density plotted over time during bacterial log growth phase. Tobramycin (at MIC) was combined with serial D-met dilutions (6400–100  $\mu\text{g/ml}$ ) and compared to clinical isolate growth. Studies were performed in triplicate. D-met did not significantly inhibit antimicrobial efficacy ( $p > 0.05$ ). Clinical isolates tested were: A) *E. coli*; B) *S. aureus*; and C) *P. aeruginosa*.



**Figure 7.**

Post Antibiotic Effect (PAE) assays of an *E. coli* clinical isolate with tobramycin (2 µg/ml). *E. coli* was exposed to tobramycin alone and in combination with serially diluted D-met (6400–400 µg/ml) for 2 hours at 37°C. Tobramycin/D-met combinations were compared to isolate and D-met (6400 µg/ml D-met and media) controls. Studies were performed in triplicate. All tobramycin/D-met growth curves were significantly inhibited ( $p < 0.05$ ) when compared with isolate controls and D-met controls.

**Table 1**

Antimicrobial interference study in normal (non-neutropenic) and neutropenic mice treated with tobramycin (n = 5/survival group; 3/bacterial recovery group) with and without D-met. The addition of D-met to tobramycin treatment did not significantly influence survival ( $p = 0.444$ ) or mean bacterial recovery ( $p = 0.197$ ).

Swiss Webster mouse <i>in vivo</i> model				
	Normal Mice		Neutropenic Mice	
Treatment	% Survival	Mean Bacterial Recovery	% Survival	Mean Bacterial Recovery
Saline Only	100	0	100	$0.5 \times 10^1$
Isolate Only	0	$8.33 \times 10^4$	80	$1.35 \times 10^5$
Tobramycin <sup>1</sup> + Isolate	60	$0.33 \times 10^1$	40	$1.17 \times 10^1$
D-met <sup>2</sup> + Isolate	0	$5.17 \times 10^4$	0	$8.47 \times 10^4$
Tobramycin + D-met <sup>3</sup> + Isolate	100	$1.75 \times 10^2$	100	$4.8 \times 10^1$

<sup>1</sup>Tobramycin dosage: tobramycin (15 mg/kg)

<sup>2</sup>D-met dosage: (200 mg/kg)

<sup>3</sup>Dosage combinations: tobramycin (15 mg/kg) + D-met (200 mg/kg)