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Adverse Outcome Pathway for Aminoglycoside Ototoxicity in Drug-Resistant Tuberculosis Treatment

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Abstract

Individuals treated for multidrug-resistant tuberculosis (MDR-TB) with aminoglycosides (AGs) in resource-limited settings often experience permanent hearing loss. However, AG ototoxicity has never been conceptually integrated or causally linked to MDR-TB patients' pre-treatment health condition. We sought develop a framework that examines the relationships between pre-treatment conditions and AG-induced hearing loss among MDR-TB-infected individuals in sub-Saharan Africa.

The adverse outcome pathway (AOP) approach was used to develop a framework linking key events (KEs) within a biological pathway that result in adverse outcomes (AO), which are associated with chemical perturbation of a molecular initiating event (MIE). This AOP describes pathways initiating from AG accumulation in hair cells, sound transducers of the inner ear immediately after AG administration. After administration the drug catalyzes cellular oxidative stress due to overproduction of reactive oxygen species. Since oxidative stress inhibits mitochondrial protein synthesis, hair cells undergo apoptotic cell death—resulting in irreversible hearing loss (AO). We identified the following pre-treatment conditions that worsen the causal linkage between MIE and AO: HIV, malnutrition, aging, noise, smoking, and alcohol use. The KEs are: (1) nephrotoxicity, pre-existing hearing loss, and hypoalbuminemia that catalyzes AG accumulation; (2) immunodeficiency and antioxidant deficiency that trigger oxidative stress pathways; and (3) co-administration of mitochondrial toxic drugs that hinder mitochondrial protein synthesis, causing apoptosis.

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This AOP clearly warrants the development of personalized interventions for patients undergoing MDR-TB treatment. Such interventions (i.e., choosing less ototoxic drugs, scheduling frequent monitoring, modifying nutritional status, avoiding poly-pharmacy) will be required to limit the burden of AG ototoxicity.

Keywords

aminoglycoside; ototoxicity; sensorineural hearing loss; tuberculosis

INTRODUCTION

Despite decades of effort to eradicate *Mycobacterium tuberculosis* (M.tb.), ~2.7 million people have been diagnosed with tuberculosis (TB) in sub-Saharan Africa—26% of the total global incidence of TB in 2015 (WHO 2016a). Particularly, multidrug-resistant TB (MDR-TB) has emerged as a global epidemic and results in significant mortality (WHO 2016a). Because MDR-TB is resistant to the powerful first-line regimens (i.e., rifampicin and isoniazid), second-line antimicrobials are used to treat this infection. Up to now, second-line regimens for MDR-TB have consisted of one injectable drug along with four or more oral anti-TB drugs. The most widely used injectable drug is an aminoglycoside (AG) given during the first phase of treatment (at least 4 months) (WHO 2016b). While the U.S. Food and Drug Administration approved AGs are gentamicin, tobramycin, amikacin, kanamycin, capreomycin, streptomycin, neomycin, and paromomycin for the treatment of serious infections caused by aerobic gram-negative bacilli, amikacin and kanamycin are the most frequently prescribed AGs globally for MDR-TB treatment recommended by the World Health Organization (WHO) (WHO 2016b).

One of the most debilitating adverse outcomes from long-term use of AGs is ototoxicity. Up to 69% of individuals with MDR-TB infection in sub-Saharan Africa experience hearing loss (Hong et al. 2018). This high incidence is almost 2-3 times higher than in high-resource countries, such as the U.S. (13%) (Marks et al. 2014), the Netherlands (18%) (de Jager and van Altena 2002), and the U.K. (28%) (Sturdy et al. 2011). AG ototoxicity appears to significantly contribute to hair cell injury, damaging both the cochlear and vestibular apparatus of the inner ear (Huth et al. 2011) (Figure 1). Typical manifestations of cochleotoxicity consist of tinnitus and/or hearing loss, which begins with high-frequency hearing loss, which may or may not be clinically apparent, and often progresses to more severe hearing loss even after discontinuation of AGs; while those of vestibulotoxicity include disequilibrium and dizziness with occasional nausea and vomiting (Ariano et al. 2008; Huth et al. 2011; Jiang et al. 2017). Vestibulocochlear impairment, moreover, can be permanent. While hearing loss is one of the most common and debilitating adverse outcomes of AGs from MDR-TB treatment, strategies to reduce the risk such as selection of less ototoxic antibiotics or systematic monitoring of hearing loss are limited in many TB programs or clinical settings in sub-Saharan Africa due to cost and constraints on human resources for health care. Compared to less-toxic antibiotics, AGs are extremely inexpensive relative to the high potency they offer (Gonzalez and Spencer 1998; Huth et al. 2011; Krause et al. 2016). Financial considerations may, in part, explain the higher incidence of AG-

induced hearing loss in resource-limited countries compared to high-resource countries. However, HIV co-infection, which is substantially more common in many low resource settings may also play a major role (Hong et al. 2018). Presently, there are no practical and cost-effective tools to identify those at highest risk for developing hearing loss from AG treatment. To avoid the unnecessary occurrence of this adverse outcome and to guide clinical decision-making, it is critical to assess individuals' potential risk for ototoxicity before initiation of MDR-TB regimen.

The Adverse Outcome Pathway (AOP) is a conceptual framework representing a set of plausible connections from an initiating event to an adverse outcome considered relevant in risk assessment in predictive toxicology (Ankley et al. 2010; OECD 2012). The framework consists of conceptual constructs and depicts existing knowledge concerning the predictive and/or causal linkages between drug initiation, physiological and molecular responses, and organ and organism-level responses (OECD 2012). Since the AOP includes integrated sequential pathways, it is often used to develop integrated tools for predictive toxicology, regulatory toxicity testing, and risk assessment (OECD 2012; Vinken 2013). Despite examination of the mechanisms of AG-induced hearing loss at a cellular level, AG-induced hearing loss has never been conceptually integrated or causally linked to MDR-TB patients' pre-treatment health condition, which may play a pivotal role in aggravating the ototoxicity pathway. Therefore, this study aimed to develop a framework that examines the relationships between pre-treatment conditions and AG ototoxicity among MDR-TB-infected individuals in sub-Saharan Africa.

MATERIAL AND METHODS

AOP Development

The conceptual framework development process was guided by the Organization for Economic Co-operation and Development's (OECD) Guidance on Developing and Assessing the Completeness of Adverse Outcomes Pathways (OECD 2012; OECD 2013). AOP methodology shares a common schematic representation consisting of a molecular initiating event (MIE), intermediate key events (KE), and an adverse outcome (OECD 2012). MIE is defined as a chemical interaction with a biological target (OECD 2012). In this study, the MIE refers to AG molecular accumulation in the interstitium of hair cells in the inner ear that initiates the toxicity pathway. The MIE is associated with a set of potential apical hazard endpoints (OECD 2012), but the AG-induced sensorineural hearing loss (SNHL) is the apical adverse outcome of interest in this pathway. The MIE and adverse outcomes are causally linked with a series of KEs that are direct chemical effects or responses initiated from or prior to the target sites through the cellular or higher levels of biological organization, scientifically proven by *in vitro* and/or *ex vivo* studies (OECD 2012). In this pathway, three key events were identified: (1) KE1, defined as prerequisite events that directly impact MIE highlighted in orange arrows; (2) KE 2, defined as prerequisite events that impact initial cellular responses highlighted in green arrows; and (3) KE 3, defined as prerequisite events that impact latter cellular responses highlighted in a purple arrow in the proposed AOP framework (Figure 3). To evaluate whether scientific qualitative and quantitative data precisely support a causal relationship between observed outcomes and a

given chemical, Weight-of-Evidence supporting the AOP was assessed by modified Bradford Hill Criteria per OECD guidelines (Hill 1965; OECD 2012). Institutional Review Board approval was not required for this study as human subjects were not involved in the research.

RESULTS

MDR-TB treatment.

Second-line injectable AGs that have been recommended by the WHO for MDR-TB treatment include amikacin, kanamycin and streptomycin (WHO 2016b); however, streptomycin is no longer considered a second-line agent because it was previously widely used for TB retreatment, and MDR-TB strains are more likely to be resistant to streptomycin than the other aminoglycosides (WHO 2016b). The selection of amikacin versus kanamycin for providers and organizations is determined by the likelihood of effectiveness, availability, and cost (WHO 2016b).

AG – Mechanism of Action.

AGs are highly potent and broad-spectrum bactericidal agents used for the treatment of serious gram-negative bacteria or mycobacteria including *M.tb.* (Mingeot-Leclercq et al. 1999). Their primary site of action is the 30S ribosomal subunit (Avent et al. 2011; Mingeot-Leclercq et al. 1999). To reach the site, molecules cross the bacterial cell wall through active transport into the cell cytosol; thereby they inhibit bacterial protein synthesis which results from misreading of the genetic code (Avent et al. 2011; Keene et al. 1982; Mingeot-Leclercq et al. 1999; Pagkalis et al. 2011). AGs have very poor oral bioavailability because they are highly polar cations. Only 0.3–1.5% of an orally or rectally administered dose of aminoglycoside reaches the systemic circulation and then appears in the urine (Pagkalis et al. 2011). Thus the route of AG administration is intravenous (IV), intramuscular (IM), intraosseous (IH), topical (cream/ointment), and ophthalmic. AGs are water-soluble and freely filtered across the glomerulus; almost all of the drug is then excreted (Avent et al. 2011; Blot et al. 2014).

MIE – Molecular interactions.

Although AGs preferentially target the bacterial ribosome, the inner ear and kidney are known to receive collateral damage (Huth et al. 2011). The mechanisms of AG uptake into sensory hair cells and renal epithelial cells increase the susceptibility to both ototoxicity and nephrotoxicity, which can be explained by the physiological similarities between the cochlea and kidney in terms of active transport of fluid and electrolytes to achieve iso-osmotic balance (Jiang et al. 2017). The accumulation of AGs appears to be dose- and duration-dependent, and uptake into the inner ear occurs rapidly and exposures persist for longer than other organs. In animal studies, AGs enter the cochlea within a few minutes and hair cells within 3 hours after systemic administration (Dai et al. 2006; Imamura and Adams 2003; Wang and Steyger 2009). AG concentrations in the inner ear are higher than plasma concentrations because the half-life of AGs in perilymph fluid are 10 to 15 times longer than in serum (Mörike et al. 1997). The receptor-mediated endocytosis at the apical surface of hair cells in the cochlea plays a role in AG uptake—AG molecules are found in vesicles

beneath the hair cells (Hashino and Shero 1995). AGs are also taken up into the renal epithelial cell line via an endocytotic process, which explains the nephrotoxicity after glomerular filtration of the agent (Hashino and Shero 1995). Along with endocytosis, the presence of several ion channels at the hair cells, such as the mechanoelectrical transducer (MET) cation channel quickens AG accumulation. The MET channel increases the potential differences between extracellular fluid and cytoplasm and functions like a one-way valve, promoting the likelihood of cellular uptake and accumulation of cationic AGs in the cytoplasm in the hair cells and renal cells (Cernada et al. 2014; Farris et al. 2004; Marcotti et al. 2005). Consequently, AG molecules accumulate rapidly and are eliminated slowly from the inner ear; thus, hair cells are more susceptible to AG-related processes than other cell types.

Cellular and Organ Responses.

Reactive oxygen species (ROS) are byproducts of normal mitochondrial metabolism; ROS contribute to organ homeostasis by controlling normal cell growth, differentiation, development, and death (Benhar et al. 2002; Zorov et al. 2014). TB infection induces ROS production through activation of phagocytes—a part of host defense mechanism against *M.tb.* (Rajopadhye et al. 2017). Further, the AG molecules that enter hair cells readily bind to cytosolic proteins, specifically calreticulin, which plays a role in Ca^{2+} homeostasis (Karasawa et al. 2010). AG binding to calreticulin dysregulates cytosolic Ca^{2+} concentration (Krause and Michalak 1997), which in turn induces mitochondrial Ca^{2+} overload, producing cytoplasmic ROS and causing mitochondrial oxidation (Esterberg et al. 2016). In addition, since AGs act as iron chelators, the formation of redox-active iron-AG complexes catalyzes oxygen-derived free radicals (Priuska and Schacht 1995; Sha and Schacht 1999b). Thus, ROS overproduction with exhaustion of the capacity of the intrinsic protective and repair system results in oxidative stress (Murphy 2013; Zorov et al. 2014). Moreover, the Transient Receptor Potential (TRP) cation channels—particularly the subfamily TRPA1 containing pore helices—are located in the outer hair cells. The TRPA1 channels function as inflammatory, irritant, and oxidative stress sensors (Henderson et al. 2006; Lesniak et al. 2005). Activation of TRPA1 channels resulting from oxidative stress or noise-exposure, enlarges the pore diameter to dimensions that are larger than AG molecules, thereby facilitating AG uptake into the hair cell (Stepanyan et al. 2011). Oxidative stress contributes to mitochondrial depolarization and dysfunction, and mitochondrial protein synthesis inhibition, which in turn activates programmed cell death-signaling pathways, such as mitogen-activated protein kinases (MAPK) (Abi-Hachem et al. 2010; Hyde and Rubel 1995; Murphy 2013; Op de Beeck et al. 2011; Shokolenko et al. 2009). Consequently, hair cells along with ancillary sensory cells and neurons—mainly the cochlear portion of the auditory nerve—undergo apoptotic cell death, resulting in irreversible SNHL (Clerici et al. 1996; Hirose et al. 1997; Priuska and Schacht 1995; Sha and Schacht 1999b).

Key Event 1: Prerequisite events that directly impact MIE

Nephrotoxicity—Individuals with renal impairment may experience decreased AG clearance and increased AG accumulation, as AGs are mostly eliminated by glomerular filtration. As a result, sustained and excessive peak serum concentrations are considered a risk factor for hearing loss. AGs are also nephrotoxic; renal function at treatment initiation

directly influences the level of AG accumulation in hair cells. Thus ototoxicity can be caused by AG toxic levels and/or renal impairment, which leads to reduced AG clearance and more drug accumulation (Huth et al. 2011). Comorbid conditions that influence renal function directly or indirectly through chronic use of nephrotoxic drugs would induce AG ototoxicity. A common example in sub-Saharan Africa is HIV co-infection. Renal complications of HIV infection are common and include proteinuria, interstitial nephritis, renal tubular damage, and nephrolithiasis; HIV-associated nephropathy, coupled with use of nephrotoxic antiretroviral drugs such as Nucleoside Reverse Transcriptase Inhibitors (NRTIs) in particular, leading to excessive AG accumulation (Calza et al. 2011; Jin et al. 2015; Kenyon et al. 2011; Kohler et al. 2009; Scherzer et al. 2012).

Pre-existing hearing loss—Pre-existing hearing loss at MDR-TB diagnosis commonly originates from previous exposure to ototoxic drugs, noise exposure, advanced age, or idiopathic SNHL (Tysome JR 2016). Particularly, because acoustic stimuli increase permeability of cation channels such as MET and TRP, the noise exposure also increases the AG uptake and directly accelerates intracellular accumulation of AGs within hair cells (Hayashida et al. 1985; Park et al. 2018; Ricci et al. 2005). Age-related hearing loss, or presbycusis, caused by the degeneration of cochlear cells is also a major cause of pre-existing hearing loss (Hu et al. 2018). As tissue ages, the hair cells also undergo progressive oxidative mitochondrial DNA damage modified by excessive ROS generation and chronic inflammatory damage due to immunosenescence (Cui et al. 2012; Iwai et al. 2008; Kalinec et al. 2017; Lee 2013; Saitoh et al. 1994). This results in auditory sensory cell degeneration. Further, HIV can cause pre-existing hearing loss directly and indirectly. It has been found that a primary HIV infection in either the central nervous system or peripheral auditory nerve causes SNHL, although the exact mechanism of nervous destruction is still unclear (Bankaitis and Keith 1995). A human study did not find histopathologic changes using electron microscopy supporting that HIV directly damages the cochlear end organs (Chandrasekhar et al. 1992). However, a recent observational study found that HIV infected adults had significantly poorer hearing threshold in both low and high frequencies than HIV uninfected adults (Torre et al. 2015a). Opportunistic infections are one of the common indirect causes of pre-existing hearing loss. The most frequent otologic opportunistic infections found in HIV infected individuals include seborrheic dermatitis of the external ear, otitis externa with otomycosis, and serous otitis media (Prasad et al. 2006; Rzewnicki et al. 2012). Because these infections are mostly caused by community-acquired organisms, such as *Pseudomonas aeruginosa, Aspergillus, fumigatus, Candida albicans* in outer ear and *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Moraxella catarrhalis* in middle ear (Prasad et al. 2006; Rzewnicki et al. 2012), frequently recurrent acute or chronic ear infections lead to conductive hearing loss before or during AG treatment (Ivanov et al. 2017). Due to lack of trained healthcare providers or devices, it is difficult to confirm SNHL by differentiating it from conductive hearing loss by comprehensive audiological assessment, including otoscopy, tympanometry, and air-bone conduction audiometry in most countries in sub-Saharan Africa (Hong et al. 2018). Thus, underdiagnosed ear infections may masquerade as AG-induced hearing loss, and undertreated ear infections aggravate oxidative stress from altered metabolic pathways (Ivanov et al. 2017). While otosyphilis is a rare complication of syphilis, it is not an uncommon cause of inner ear infection in people

living with HIV. A clinical manifestation of acute syphilis with cochleovestibular involvement includes sudden SNHL (Weder et al. 2013); otosyphilis amplifies oxidative stress but thereby may magnify symptoms of AG-induced hearing loss. Otosyphilis seems to lead to endolymphatic hydrops in the cochlea and/or atrophy of the organ of Corti, spiral ganglion, and stria vascularis (Miller et al. 2010) (Figure 2), which may reduce endocochlear potential, resulting in cochlear sensitivity to sound. Also, because HIV drugs, particularly NRTIs—including zidovudine, didanosine, stavudine, lamivudine—have ototoxic potential via their effect of reducing mitochondrial DNA content, use of NRTIs prior to MDR-TB treatment may potentiate the ototoxic effect of AGs (Simdon et al. 2001a; Torre et al. 2015b). This association has been specified in key event 3 (Figure 3).

Hypoalbuminemia—Malnutrition—an insufficiency or unbalance of nutrition (Kelly et al. 1999)—is a significant health issue in people living with MDR-TB with or without HIV (Ivers et al. 2009; WHO 2003b) and is more prominent in resource-limited environments due to food insecurity (HealthyPeople.gov ; WHO 2012). Malnutrition is a result of a deficiency of both macronutrients (nutrients that provide calories or energy, including carbohydrates, proteins, and fat) and micronutrients (i.e., vitamins and minerals), vital dietary components necessary for physical and mental development, disease prevention, and well-being (de Pee and Semba 2010; WHO 2003a). Most individuals with active TB are in a catabolic state and experience weight loss and signs of vitamin and mineral deficiencies (Mohamed-Hussein et al. 2016). Protein-energy malnutrition (PEM) caused by insufficient intake of protein and calories is more prominent among TB and HIV co-infected patients and is worsened by TB-induced muscle wasting (Anema et al. 2009; de Pee and Semba 2010; Hood 2013; Koethe et al. 2009; van Lettow et al. 2003). In the case of PEM, albumin synthesis is impaired, leading to low serum albumin concentration (i.e., hypoalbuminemia) (Bisaso et al. 2014; van Lettow et al. 2003). Since albumin plays a pivotal role in maintaining colloid oncotic pressure, hypoalbuminemia results in abnormal increase of inner ear fluid volume by diminishing the osmotic gradient (Kim et al. 2017; Kim et al. 2011), accelerating AG accumulation because AG is water-soluble (Blot et al. 2014).

Key Event 2: Prerequisite events that impact initial cellular responses

Immunodeficiency—HIV infection weakens the human immune system by killing T-helper cells, macrophages, and dendritic cells, thus causing immunodeficiency (Freed and Martin 2007). HIV infection leads to chronic activation of nuclear factor (NF)-κB—a master regulator of pro-inflammatory genes, which produces pro-inflammatory cytokines, such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α) (Fiume et al. 2012). Soon after secretion of pro-inflammatory cytokines, neutrophils and other immune cells migrate to the infection site in various cell types depending on opportunistic infections including TB, where they ingest bacterium and kill them by releasing ROS, which causes oxidative stress and mitochondrial DNA damage (Rajopadhye et al. 2017). Further, HIV-infected individuals with advanced disease have increased levels of oxidative DNA damage biomarkers (i.e., 7,8-dihydro-8-oxoguanine) in CD4+ T cells and show declines in DNA glycosylase activity for the repair of oxidative base lesions in these cells (Aukrust et al. 2005). In addition, the number of CD4+ cells is positively associated with the levels of intracellular concentration of antioxidants, especially glutathione (Staal et al. 1992; Wanchu

et al. 2009). In particular, people living with HIV who are not taking ART may have increased risk of AG ototoxicity since ART restores the numbers of CD4+ T-cells while it augments the imbalanced redox status (Elias et al. 2013).

Antioxidant deficiency—Antioxidant deficiency causes hair cells to be more vulnerable to oxidative stress, which contributes to apoptotic hair cell death. For example, the presence of glutathione—an endogenous antioxidant resulting in detoxification of xenobiotics and protection against ROS (Marí et al. 2009)—protects the hair cells against oxidative stress (Garetz et al. 1994; Nishida and Takumida 1996). Several studies also found that dietary nutrient-based antioxidant supplementation, including vitamin A, β-carotene (one of the provitamin A carotenoids), vitamin C, and vitamin E, significantly attenuated outer hair cell damage, as they have anti-inflammatory properties (Aladag et al. 2016; Clerici et al. 1996; Le Prell et al. 2014). Albumin also has antioxidant properties through its multiple binding sites and capacity to trap free radicals (Roche et al. 2008). Thus, hypoalbuminemia also worsens antioxidant deficiency. Antioxidant deficiency is caused not only by poor intake of dietary sources but also by smoking, alcohol consumption, and aging which inhibit synthesis of antioxidant enzymes and reduce antioxidant concentrations (Albano 2006; de Grey 1999; Donohue 2006; Isik et al. 2007; Khare et al. 2014; Lemasters 2005; Sitar et al. 2013).

Key Event 3: Prerequisite events that impact latter cellular responses

Mitochondrial toxicity—Use of mitochondrial toxic drugs may potentially worsen AG ototoxicity. NRTIs can inhibit human DNA polymerases, including gamma polymerase—important to mtDNA replication—that may damage mtDNA; key event 3 may also directly activate the MAPK pathway (Marra et al. 1997; McNaghten et al. 2001; Simdon et al. 2001b). In particular, Tenofovir Disoproxil Fumarate (TDF) is one of the most common choices of NRTI; however, it targets the mitochondria of both hair cells and renal proximal tubules, increasing the risk of ototoxicity, as does key event 1. Since the combination of two NRTIs constitutes the backbone of ART regimens (Bartlett et al. 2012), individuals on both NRTIs and AGs are at higher risk of apoptotic hair cell death.

Assessment and Confidence Testing

Bradford Hill Criteria consist of 6 items, evaluating the concordance, strength, consistency, and specificity of associations between conceptual constructs within AOP, as well as the biological plausibility and coherence of experimental evidence (OECD 2012). To achieve confidence in the proposed AOP, 5 items were addressed to evaluate the mechanistic understanding of biological systems (OECD 2012).

Assessment of the AOP According to the Bradford Hill Criteria (Hill 1965)

Concordance of dose-response relationships: AG-induced ototoxicity occurs in basal outer hair cells and then extends to inner hair cells and further apical outer hair cells with increasing cumulative AG dose (Jiang et al. 2017). Many classic laboratory animal studies have revealed that AG-induced vestibulocochlear toxicity ranges over duration and levels of exposure (Aran and Darrouzet 1975; Lenoir and Puel 1987; Michaels 2012; Wersall and Hawkins 1962). Cochleotoxicity was tested in response to a range of amikacin doses in adult rats for 5 consecutive days (Lenoir and Puel 1987). They found that hair-cell stereocilia

degeneration occurred in the high-dose group (i.e., 600-1000 mg/day),while the low-dose group did not develop cochlear abnormalities (i.e., 200 mg/day) (Lenoir and Puel 1987). In addition, the pattern of hair cell degeneration—most severe in the basal regions of the cochlea with decreasing gradient towards the apex—was dependent on the administered dose of amikacin (Lenoir and Puel 1987). Streptomycin also causes vestibulotoxicity in a dose-response manner. Vestibular disturbance was observed in cats 12-19 days after receiving a low-dose of streptomycin (100-200 mg/kg daily); however, the cats that received 400 mg/kg became ataxic shortly after administration of the first dose, which persisted for almost 24 hours (Wersall and Hawkins 1962).

Temporal concordance among the key events and adverse outcome: The temporal relationship among the three key events are dependent on the pre-treatment conditions that are present. Each pre-treatment factor may influence multiple key events that occurs in sequential order. Prerequisite events are mediated by presence of pre-treatment factors with or without exposure to toxicants, which preceded AG accumulation in the interstitium of hair cell (MIE). Since AG accumulation is an essential prerequisite for apoptosis of hair cells, the temporal sequence from pre-treatment conditions through AG induced SNHL is well supported.

Strength, consistency, and specificity of association of adverse outcome and initiating event: We explained that AG-induced SNHL is caused by the oxidative stress that results from excessive AG accumulation in the hair cells (MIE). The causality of this pathway can be inversely proven by the following experimental and clinical studies that tested protective effects by targeting various steps of the ototoxic cascades:

(1) Reducing AG uptake: Evidence for the molecular identity of the MET channel strongly supports the potential modification of MET channel permeability, reducing AG uptake (Corns et al. 2017; Farris et al. 2004; Kirkwood et al. 2017).

(2) Iron chelators and antioxidants as ROS scavengers: Attenuated hair cell apoptosis capacity have been confirmed by administration of iron chelators or antioxidative agents, such as salicylates (Lecain et al. 2007; Sha and Schacht 1999a), deferoxamine (Mostafa et al. 2007), N-Acetylcysteine (Aladag et al. 2016; Garcia-Alcantara et al. 2017), D-Methionine (Campbell et al. 2016; Fox et al. 2016), α-lipoic acid (Wang et al. 2012), ascorbic acid (vitamin C) (Le Prell et al. 2014; Wu et al. 2015), α-tocopherol (vitamin E) (Fetoni et al. 2004; Le Prell et al. 2014), magnesium (Le Prell et al. 2014), and misoprostol (Dogan et al. 2017).

(3) Inhibiting the MAPK pathway: Inhibition of the MAPK pathway by application of D-JNKI-1 (Wang et al. 2003), CEP 11004 (Bodmer et al. 2002), and estradiol (Nakamagoe et al. 2010) prior to AG administration resulted in significant protection from hair cell death in vitro and hearing loss in vivo.

Biological plausibility, coherence, and consistency of the experimental evidence: The biological plausibility, coherence, consistency, and strength of the experimental evidence that supports the proposed AOP is detailed in Table 1.

Alternative mechanism(s) that logically present themselves and the extent to which they may distract from the postulated AOP: The mechanism of AG-induced ototoxicity with hearing loss is less understood. However, one potential alternative hypothesis is the presence of N-methyl-D-aspartate (NMDA) at the synapse between cochlear hair cells and spiral ganglion neural afferents (Basile et al. 1996; Puel et al. 1991). At NMDA receptors, AG mimics the positive modulation of polyamines, potentially leading to excitotoxic damage at the hair cell-afferent nerve synapses (Choi 1992). Since hair cell apoptosis resulted from ROS overproduction is a significant modifiable pathogenesis of AG ototoxicity, our AOP did not include mechanisms of NMDA receptors, and thereby separate AOP could depict such alternative mechanism.

Uncertainties, inconsistencies and data gaps: Assessments of human tissue from patients with MDR-TB, with or without HIV co-infection, for evidence of AG-related pathophysiology have not been conducted for obvious ethical reasons. Although AG ototoxicity has been comprehensively studied, the major events within the proposed AOP have been causally explained by healthy preclinical animal models, while AG has mostly been administered to those with severe infections in clinical settings. However, a recent animal study has induced systemic host-mediated inflammatory conditions by injecting lipopolysaccharide (LPS), an important component of bacterial endotoxin to experimental mice (Hirose et al. 2014; Koo et al. 2015). While LPS alone did not affect hearing, mice that received LPS prior to ototoxic agents had worse hearing loss than those that did not receive LPS pretreatment resulted from accelerated AG uptake (Hirose et al. 2014; Koo et al. 2015). Such animal studies are unable to fill the gap entirely, but evidence from preclinical work supports the hypothesis that persistent inflammation contributes to AG ototoxicity.

Confidence in the AOP

How well-characterized is the AOP?: AG-induced ototoxicity is a well-understood phenomenon. We adapted the *Mitochondrial Free Radical Theory of Aging* to explain the relationship between AG molecules and active free radicals, which are generally produced in the organism, at the cellular level (de Grey 1999). Such relationship is supported by experimental data, as specified in Table 1.

How well are the initiating and other key events causally linked to the outcome?: Multiple experiments demonstrated that AGs are causally linked to SNHL in a dose-dependent way in both animals and humans. Evidence is strong to support a causal relationship between each key event and SNHL.

What are the limitations in the evidence in support of the AOP?: There are unmeasurable variables that may confound the relationship outlined in the AOP, such as known and unknown genetic mutations or additional confounders we may not have thought of. Specifically, mtDNA mutation is a risk factor that may be considered as one of the pre-treatment conditions as several genetic mutations also increase the susceptibility to ototoxicity. The mitochondrial rRNA mutation, particularly in the 12S rRNA gene, such as A1555G (most common), C1494T, T1095C, T1291C, 961delT+C(n), and A827G, among others, increase the structural similarity of human mitochondrial ribosomal RNA (rRNA) to

bacterial 16S rRNA (Hamasaki and Rando 1997; Hobbie et al. 2008; Prezant et al. 1993). As a result, mutated mitochondrial ribosomes in the cochlea become target-binding sites for AGs (Cox et al. 1964; Davies et al. 1965), and AGs lead to misreading of the genetic code along with perturbation of ribosomal translation (Hamasaki and Rando 1997; Hobbie et al. 2008). This causes mitochondrial ribosomal damage and further cytotoxicity as it directly activates the MAPK pathway with apoptosis (Benhar et al. 2001; Owens et al. 2007; Son et al. 2013; Wang et al. 2003). The most common type of mitochondrial A1555G gene mutation is most prevalent in Europeans (0.19%) (Bitner-Glindzicz et al. 2009; Vandebona et al. 2009) but not in Sub-Saharan Africans, where the prevalence of the mutation is extremely low (0% to 0.09%) (Bosch et al. 2014; Kabahuma et al. 2011; Lasisi et al. 2014; Wonkam et al. 2015). As a result, mtDNA mutation was not addressed and generalizability is limited because this model targets evidence obtained within the Sub-Saharan African MDR-TB populations and in resource-limited settings. To date, numerous experimental studies in this area are ongoing, so new evidence may change this AOP.

Is the AOP specific to certain tissues, life stages/age classes?: Advanced age may increase the risk for AG ototoxicity. Presbycusis is difficult to characterize because of genetic and environmental influences, and because of its complexity of structural changes confounded by various medical, psychological, and pharmacologic factors (Patel and McKinnon 2018). However, presbycusis is also caused by apoptotic hair cell death resulting from excessive oxidative cellular stress, which in turn stimulates the MAPK pathway. Age-dependent renal function is also closely related to this pathway because AG elimination is mostly completed through renal clearance. Age-related reduction in creatinine clearance among elderly populations increase the risk of ototoxicity (Fraisse et al. 2014; Weinstein and Anderson 2010). The glomerular filtration rate is low at birth, reaches about adult levels by the end of the second year of life, and declines after the fourth decade (Weinstein and Anderson 2010). Thus, infants, young children, and the elderly are more susceptible to AG-induced SNHL, but this AOP is developed targeting adult populations.

Are the initiating and key events expected to be conserved across taxa?: Experimental studies in multiple types of animals across species, including zebrafishes (Kim et al. 2018; Wang and Steyger 2009), bullfrogs (Dai et al. 2006), chicks (Dai et al. 2006; Hashino and Shero 1995), mice (Bächinger et al. 2018; Corns et al. 2017; Dai et al. 2006; Hirose et al. 2014; Koo et al. 2015; Yu et al. 2018), rats (Garcia-Alcantara et al. 2017; Ladrech et al. 2017; Lenoir and Puel 1987), turtles (Farris et al. 2004), cats (Wersall and Hawkins 1962), and guinea pigs (Aran and Darrouzet 1975; Bareggi et al. 1990; Campbell et al. 2016; Dai et al. 2006; Garetz et al. 1994; Hayashida et al. 1985; Imamura and Adams 2003; Tunstall et al. 1995)—all show evidence in support of this pathway. Human autopsies have also shown this relationship (Keene et al. 1982; Nordstrom et al. 1990).

DISCUSSION

Overall, AG ototoxicity caused by apoptotic hair cell death is a complex process, although our understanding of it has increased in recent years. Based on the modified Bradford Hill Criteria, we believe this AOP provides critical, evidence-based insights into AG-induced hearing loss. AG-induced hearing loss prevention in TB programs is a real challenge due to

complicated clinical conditions, and the causal relationship between treatment and adverse outcomes is often difficult or impossible to determine definitively. Although maintaining therapeutic, but not supra-therapeutic, AG concentration aids in hearing loss prevention and cure of MDR-TB, frequent therapeutic drug monitoring (TDM) is impractical in most resource-limited settings. While the causative genomic variants have been studied to determine the phenotype-genotype correlations with AG-induced hearing loss (Alford et al. 2014), genetic services are not available in many clinical settings as a screening tool. As there are no practical screening tools to aid in the prevention of ototoxicity, knowing the mechanism of AG ototoxicity and its linkage with pre-treatment physical conditions associated with MDR-TB is critical for designing strategies to prevent AG-induced irreversible SNHL.

This is the first attempt to develop an AOP framework that outlines the apoptotic cascade in AG toxicity. This AOP framework will broaden our understanding of the complexity of AG-induced hearing loss and interactive health conditions in individuals before and after AG exposure. Such schematic representations can be used as a tool for healthcare providers to make clinical decisions, particularly in developing personalized interventions, such as choosing less ototoxic drugs or scheduling more frequent toxicity monitoring. The proposed AOP can be favorably applied not only in clinical practice but also widely in public health research as it is helpful in hypothesizing the relationships between different covariates associated with drug-induced adverse outcomes. Examples of clinical implications and recommendations based on the key elements and contributors to hearing loss are summarized in Table 2.

Since AG ototoxicity is concentration-dependent, AG dose and use should be tightly regulated in inpatient settings, with serial measurement of creatinine and estimation of creatinine clearance coupled with TDM, which is a measurement of aminoglycoside peaks and troughs, and adjustment of dosing to remain in the targeted therapeutic ranges (Avent et al. 2011). In outpatient settings or home-visiting programs; however, optimizing AG dosing is considerably challenging because TDM is unavailable in real time. As a result, detection of ototoxicity could be delayed because cochlear damage is initially asymptomatic. Thus, future research should consider to develop a surrogate measure of AG concentration without laboratory testing and examine its practical feasibility in resource limited environment.

Although the proposed AOP is theoretically and practically useful, application is limited to MDR-TB treatment in resource-limited settings particularly in sub-Saharan Africa because this study does not take account for genetic variance. Furthermore, we acknowledge that the proposed AOP oversimplifies the complex pharmacopathological and pharmacotoxicological process, which did not capture all potential mechanisms. Since this AOP was developed based on currently available scientific evidence, it must be considered an open and flexible framework that requires continuous refinement. There is a need for well-designed and adequately powered observational studies to identify the risk factors for AG ototoxicity that are present at MDR-TB treatment initiation and during treatment, through thorough history taking and frequent hearing screening. Since polypharmacy is common among people with MDR-TB and HIV (Alomar 2014), future studies may be helpful in elucidating drug-drug interactions and drug-gene interactions with AG and would be a good scientific addition to

understanding and prevention of AG-induced hearing loss. Continuous attention to the prevention of AG-induced hearing loss during MDR-TB treatment is critical not only in resource-limited settings but also as global policy.

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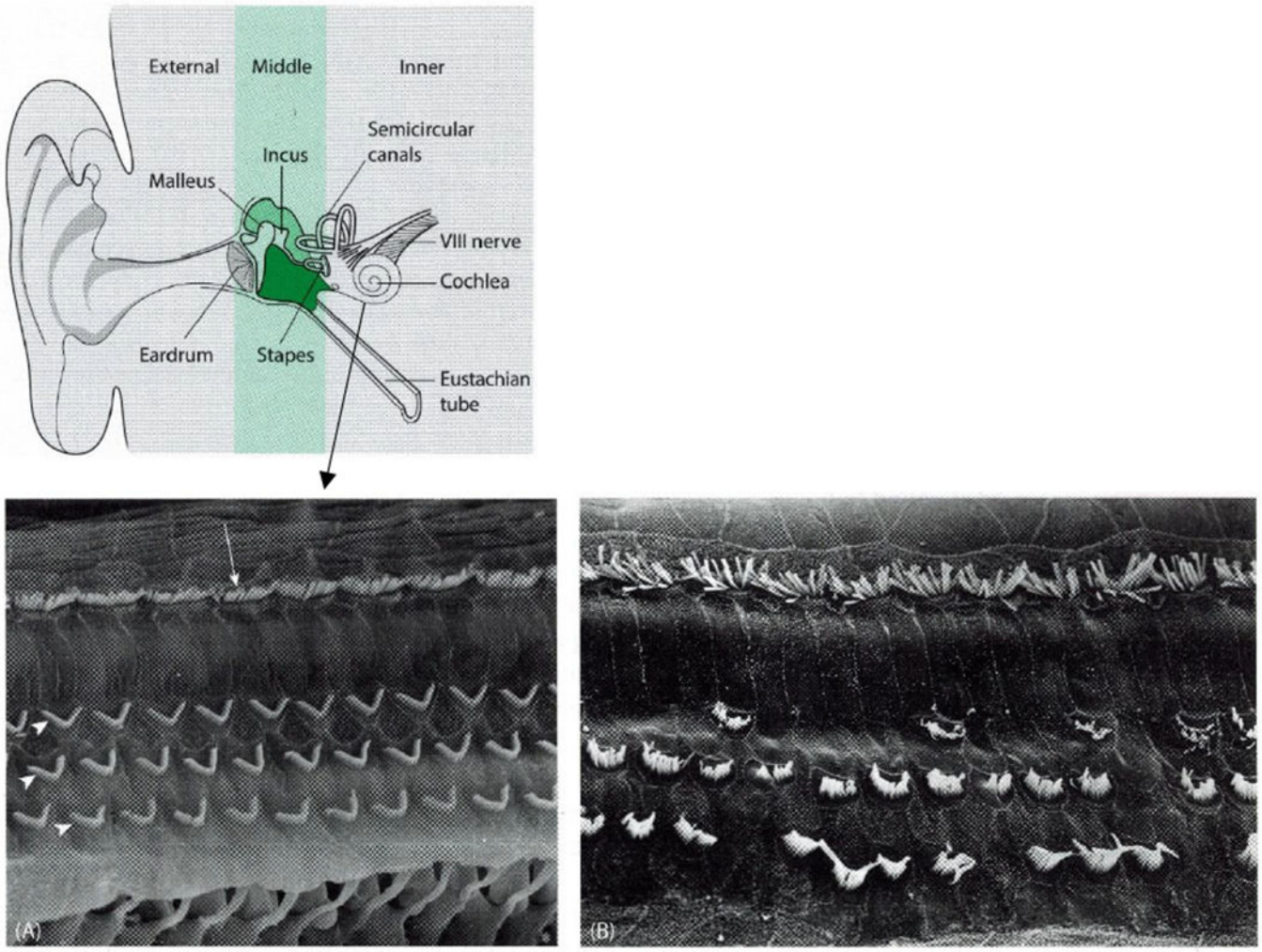

Figure 1.

Anatomy of inner ear and hair cells

(A) Electron micrograph of normal outer (arrowheads) and inner (arrow) cochlear hair cells; (B) Electron micrograph of damaged cochlear hair cells. This illustration and image were adapted with permission from Taylor & Francis Group, LLC. (Tysome JR 2016)

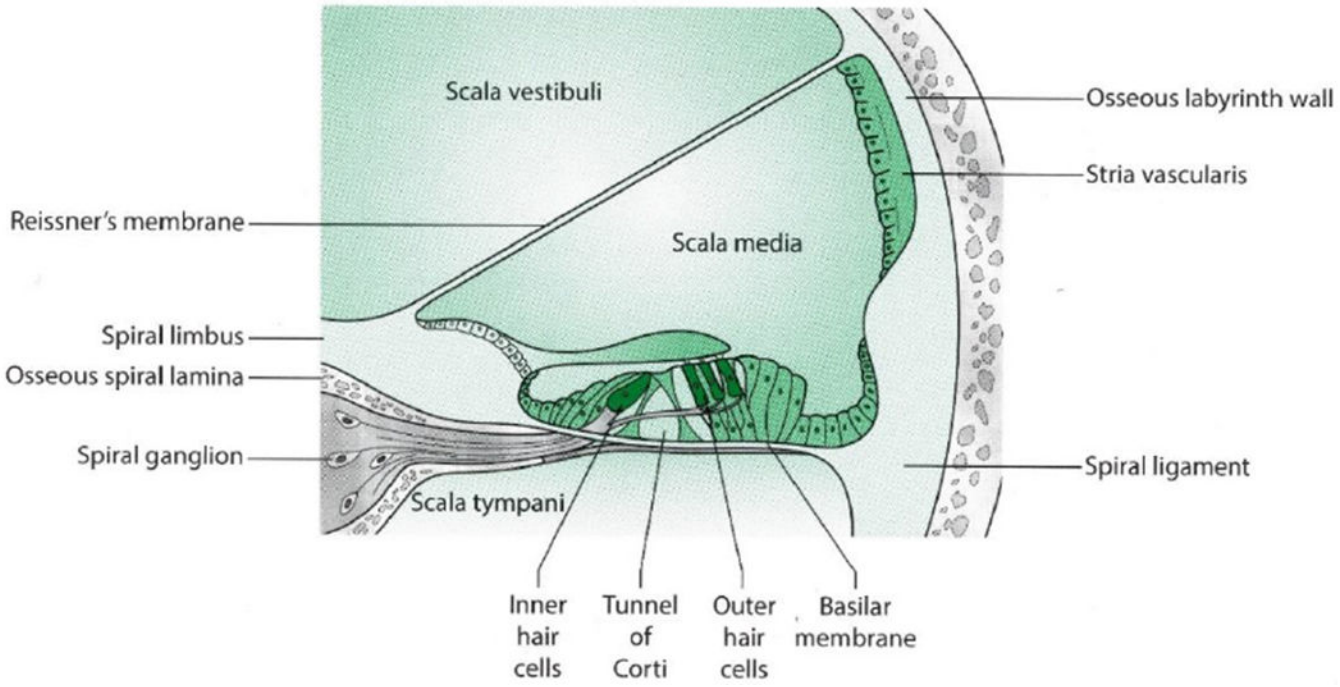

Figure 2.

Cross-sectional view of the cochlear duct

Diagram not to relative scale. This illustration was adapted with permission from Taylor & Francis Group, LLC. (Tysome JR 2016)

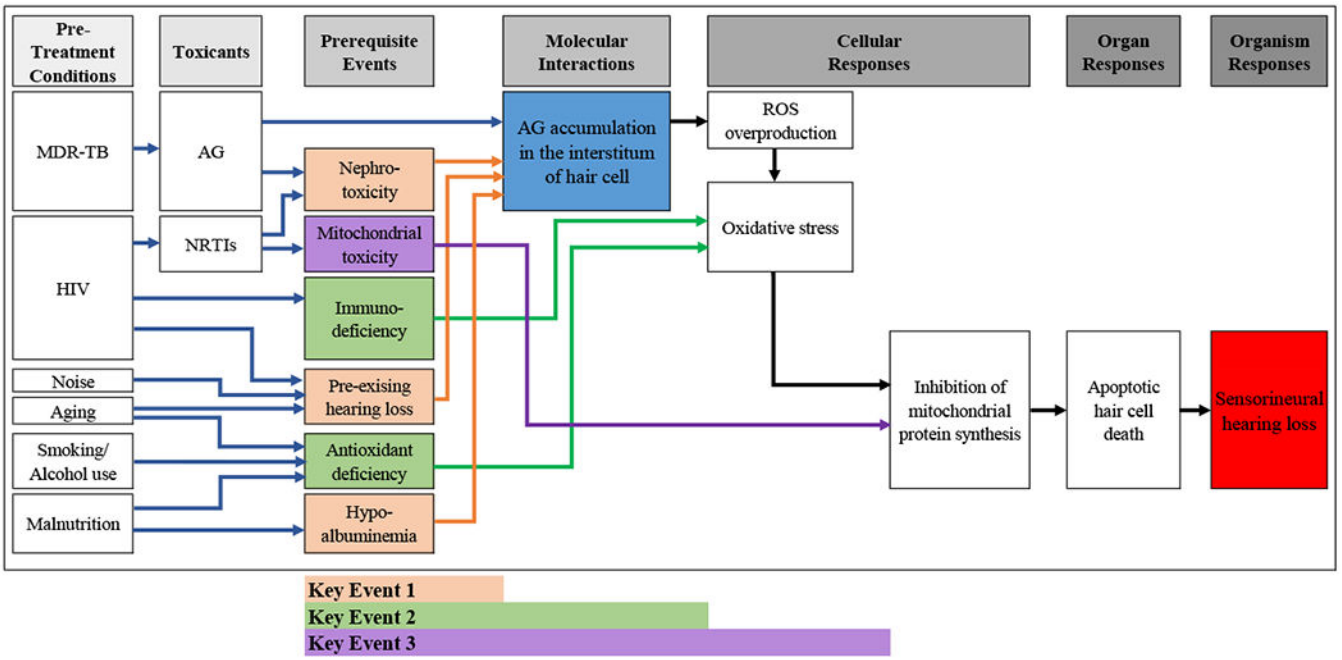

Figure 3.

Conceptual framework of Adverse Outcome Pathway on AG ototoxicity in MDR-TB treatment

Abbreviations: AG= aminoglycoside; AO= adverse outcome; HIV= human immunodeficiency virus; KE= key event; MDR-TB= multidrug-resistant tuberculosis; MIE= molecular initiating event; mt= mitochondrial; NRTI= Nucleoside Reverse Transcriptase Inhibitor; ROS= reactive oxygen species; SNHL= sensorineural hearing loss

Table 1.

Summary of Information on the Key Events of the AOP

Key Events	Description of Events	Experimental Support and References
MIE: AG accumulation in the inner ear hair cell	The receptor-mediated endocytosis and presence of MET cation channel lead to rapid accumulation and slow elimination of AG in the inner ear	(1) KM was taken up into sensory hair cells via receptor-mediated endocytosis at their apical surfaces because AG molecules are found in vesicles beneath the hair cells from White Leghorn chicks, confirmed by immuno-gold electron microscopy (Hashino and Shero 1995; Richardson et al. 1997) (2) MET channels on hair cell functions as open transducer channels that is the main route for aminoglycoside entry. AGs functioned as voltage-dependent MET channel blockers that also rapidly permeate through MET channels into hair cells, which was found in bullfrog model (Steyger et al. 2003), turtle model (Farris et al. 2004), and mouse model (Marcotti et al. 2005). The AG molecules enter the channel and block the ion-conducting pathway, thus such blockage increases voltage. Increased AG entry through the channel pore into the hair cell due to the large electrical driving force also increases the affinity for the blocker. This boosts both the entry of AG into the channel and the channel's affinity for the drug (Farris et al. 2004; Marcotti et al. 2005; Steyger et al. 2003).
KE 1: Prerequisite events impacting MIE	Nephrotoxicity (KE1-1), hypoalbuminemia (KE1-2), and pre-existing hearing loss (KE1-3) accelerate AG accumulation in the interstitum of hair cell	(1) TDF is mitochondrial toxic, increasing number of abnormal mitochondria including irregular mitochondrial shape, and sparse, fragmented cristae. Abnormal proximal tubule functioning and decreased GFR occurred in patients who had been taking TDF in multiple studies (Calza et al., 2011; Jin et al., 2015; Kenyon et al., 2011; Kohler et al., 2009; Scherzer et al., 2012). (2) The albumin-like proteins, including albumin, IgG, IgA, transferrin, antitrypsin, and haptoglobin were the major protein compositions of luminal fluid in inner ear (Kim et al. 2017; Kim et al. 2011). Among patients (n=11) with enlarged vestibular aqueducts, patients with recent hearing loss and increased volume of luminal fluid showed a significantly decreased proportion of the albumin-like proteins in the interstitial space (Kim et al. 2011). (3) Pre-existing hearing loss includes mainly noise-induced and age-related hearing loss. Histological evaluation using mice (n=22), received repetitive exposure the acoustic stimuli (~4.5 kHz with 120.5 dB sound pressure level), showed significant structural hair cell damage (Park et al., 2018). It has been found that atrophy of the stria vascularis of cochlear duct was observed in older mice (older than 12 months at least), and that lipofuscin (aging associated pigment granules) accumulation in inner and outer hair cells of the mice were also found from the same age (Saitoh et al. 1994). This structural change is accelerated by the age-related dysfunctions of the systemic immune system accelerated, worsening presbycusis (Cui et al. 2012; Iwai et al. 2008; Kalinec et al. 2017; Lee 2013; Saitoh et al. 1994).
KE 2: Prerequisite events impacting initial cellular responses	Immunodeficiency (KE2-1) and antioxidant deficiency (KE2-2) trigger cellular oxidative stress	(1) In comparison between 8 HIV-infected patients (mean CD4+ T-cells count = 280×10^6/L), 7 AIDS patients (mean CD4+ T-cells count = 45×10^6/L), AIDS patients had increased levels of 7,8-dihydro-8-oxoguanine in CD4+ T cells and marked declines in DNA glycosylase activity for the repair of oxidative base lesions in these cells (Aukrust et al. 2005). People living with HIV showed elevated pro-inflammatory cytokines, including interleukin 2 (IL-2), IL-6, and tumor necrosis factor-alpha (TNF-α) and biomarkers associated with inflammation and coagulation, including C-reactive protein (CRP) and D-dimer due to chronic inflammation and immune activation (Abrams et al. 2009; Baker et al. 2010; Deeks et al. 2013; Neuhaus et al. 2010). In cross-sectional human study, the level of lipid peroxidation (LPO) and glutathione were used as a means of determining oxidative stress. The mean LPO levels were significantly higher in HIV-infected patients (n=100; mean= 0.7 ± 0.1 μmol/ml) as compared to healthy controls (n=30; mean= 0.3 ± 0.1 μmol/ml). The mean glutathione level in HIV-infected patients (0.06 ± 0.01 μmol/ml) was significantly lower in compared to healthy controls (0.09 ± 0.01 μmol/ml) (Staal et al. 1992; Wanchu et al. 2009). (2) A human study found that compare to younger control subjects, elderly subjects had significantly lower level of glutathione (2.08 ± 0.12 vs. 1.12 ± 0.18 mmol/L RBCs; $P < 0.05$); glutathione synthesis rates (1.73 ± 0.16 vs. 0.55 ± 0.12 mmol/L RBCs per day; $P < 0.01$); and higher plasma oxidative stress (304 ± 16 vs. 346 ± 20 Carratelli units; $P < 0.05$) simultaneously (Sekhar et al. 2011). This indicates that glutathione deficiency in elderly humans resulted from a marked reduction in synthesis, related to oxidative stress.

Key Events	Description of Events	Experimental Support and References
KE 3: Prerequisite events impacting latter cellular responses	Mitochondrial toxicity (KE3) worsens inhibition of mitochondrial protein synthesis of hair cells	Since reductions in mitochondrial DNA content induced by NRTIs, significantly more HIV-infected patients had or developed persistent hearing loss with/without tinnitus during follow-up (Marra et al. 1997; McNaghten et al. 2001; Simdon et al. 2001a). After short-term exposure to AZT, d4T, ddC, ddI, and FLT (6-72 hours), mtDNA copy numbers were markedly decreased because the NRTIs inhibit mtDNA replication (Smith et al. 2017). Upregulation of glutathione S-transferase 4 expression were significantly increased, which suggests that ROS defense mechanisms likely to be induced by NRTI administration due to mtDNA intoxication (Smith et al. 2017).
AO: SNHL	When programmed cell death-signaling pathways has been activated, hair cells, ancillary sensory cells, and neurons undergo apoptotic cell death, resulting in irreversible SNHL	ROS formation through ototoxicants, including gentamicin and kanamycin, in cochlear tissues of was directly observed in guinea pig by electron paramagnetic resonance spectrometry (Clerici et al. 1996) and in chick by using dichlorofluorescin (Hirose et al. 1997). When chicks and mouse cochlear and vestibular hair cells were exposed to gentamicin, the incorporation of methionine-free medium over 24 hours was reduced by 30–60% compared to control conditions observed by fluorescence microscopy (Francis et al. 2013). This indicates gentamicin inhibited the medium uptake into hair cells by inhibiting protein synthesis in hair cells and activate a c-Jun N-terminal kinase (JNK) pathway as JNKs activate apoptotic signaling (Francis et al. 2013).

Abbreviations: MIE= molecular initiating event; KE= key event; AO= adverse outcome; HIV= human immunodeficiency virus; CD4= cluster of differentiation 4; SNHL= sensorineural hearing loss; MET= mechanoelectrical transducer; NRTI= Nucleoside Reverse Transcriptase Inhibitor; KM= kanamycin; TDF= Tenofovir disoproxil Fumarate; ROS= reactive oxygen species; AZT= zidovudine (3’-Azido-3’-deoxythymidine); d4T= stavudine (2’,3’-didehydro-2’,3’-deoxythymidine); ddC= zalcitabine (2’,3’-dideoxycytidine); ddI= didanosine (2’,3’-dideoxyinosine); FLT= alovudine (3’-deoxy-3’-fluorothymidine)

Table 2.

AOP implications and recommendations in MDR-TB treatment

Pre-treatment conditions or prerequisite events	Recommendations
Untreated HIV and/or NRTI use	• Monitor CD4+ T-cell count and viral load. • Consider NRTI-sparing antiretroviral regimen. • Monitor oto- and nephro-toxicity more closely.
Renal insufficiency	• Monitor renal function more closely including BUN, creatinine (serum or urine), and creatinine clearance, etc. • If HIV-infected, consider NRTI-sparing antiretroviral regimen and avoid other nephrotoxic agents
Antioxidant deficiency Hypoalbuminemia	• Provide dietary counseling. • Consider macro- and micronutrient supplementation. • Monitor serum albumin level more closely.
Pre-existing SNHL	• Conduct comprehensive audiologic evaluations including occupational/recreational noise exposure, family history of ototoxicity or hearing loss, audiometry, tympanometry, and otoscopy prior to AG initiation. • If moderate to severe hearing loss screened consider AG-sparing MDR-TB regimen or more frequent systematic audiologic evaluations should be followed.
Substance abuse	• Provide alcoholism and smoking cessation counseling and rehabilitation

Abbreviations: AG= aminoglycoside; CD4= cluster of differentiation 4; HIV= human immunodeficiency virus; SNHL= sensorineural hearing loss; BUN= blood urea nitrogen; MDR-TB= multidrug-resistant tuberculosis; NRTI= Nucleoside Reverse Transcriptase Inhibitor