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Strategies for Reducing or Preventing Ototoxic Side Effects of Aminoglycoside Antibiotics in Live-stranded Odontocetes: A Review

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Romy Boiskin is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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2014

DEDICATION

This thesis is dedicated to:

Juliette Nash, without whom I never would have began my internship in the lab;

Ann Bowles, without whom I would not have considered pursuing this degree;

and Sully the pilot whale, the inspiration for this project.

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ABSTRACT OF THE THESIS

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by

Romy Greer Boiskin

Master of Science in Biology

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Aminoglycoside antibiotics (AGs) are used to treat infections in live-stranded odontocetes. Although AGs have high clinical efficacy, they can be ototoxic, especially to high frequency hearing. This hearing loss is often irreversible and warrants concern because it is likely to affect odontocete echolocation and communication. AGs have a low toxicity threshold and thus a narrow therapeutic window. Common terrestrial mammal dosing protocols could be inappropriate for odontocetes because they may have

a reduced ability to clear AGs. Poor health, common in stranded odontocetes, compounds this problem. It may also compromise intrinsic mechanisms that maintain homeostasis and postpone cochlear hair cell death. These factors can further narrow the therapeutic window, causing otherwise safe drug levels to become toxic. There are several effective ways of attenuating AG ototoxicity such as minimizing the area under the concentration-time curve by extending dosage intervals. Therapeutic monitoring and additive therapies can additionally reduce ototoxicity. Until ototoxic risk and risk reduction strategies are explored in odontocetes, it is safest to test a stranded animal's hearing prior to its release. Hearing loss may only present weeks after treatment cessation due to AG cytotoxicity characteristics and compensatory mechanisms thereof. AGs have a protracted cochlear elimination half-life that may cause hearing loss to worsen for months after treatment. Developing strategies for reducing ototoxic risk, determining prevalence of ototoxicity in stranded animals, and documenting the functional effects of hearing loss would make it easier to estimate the odds of post-release survival in AG-treated odontocetes.

Section 1: Introduction

The first aminoglycoside (AG) antibiotic and its associated toxicities were discovered almost seven decades ago. Since then, no drug class has been discovered that is broadly as clinically applicable or outcompetes them in cost to efficacy ratio (Forge and Schacht 2000, Arya 2007). However, as indispensable as these antibiotics are clinically, they carry a relatively high risk of side effects. These include cochlear toxicity (hereafter ototoxicity), vestibulotoxicity, nephrotoxicity and neuromuscular blockade.

Reports since the discovery of AGs have shown that they are ototoxic to a variety of mammals and other animal types (Xie *et al.* 2011). It is possible that marine mammals are similarly susceptible to AG ototoxicity, but there is a paucity of data regarding effects of AGs on marine mammals including odontocetes. Although technological advances have made it easier to test odontocete hearing in recent years, there are still very few published AG ototoxicity studies (Finneran *et al.* 2005b, Montie *et al.* 2011, Schlundt *et al.* 2011). Recently, AG ototoxicity was strongly implicated as the cause of hearing loss in a beluga (*Delphinapterus leucas*) indicating that odontocetes are likely susceptible to AG ototoxicity (Finneran *et al*. 2005b). Additional cases since then have attributed AG ototoxicity as a possible cause of hearing loss in other odontocete species (Houser and Finneran 2006, Pacini *et al*. 2010, Greenhow *et al*. 2011, Schlundt *et al.* 2011).

AGs are commonly used in live-stranded odontocete cetacean (toothed whale, dolphin or porpoise) rehabilitation (Dierauf and Gulland 2001, Mann *et al.* 2010, Pacini *et al*. 2010, Greenhow *et al*. 2011, Montie *et al.* 2011, Schlundt *et al.* 2011). They are used to treat a variety of infection types, including antibiotic-resistant and virulent strains

of bacteria (Schacht 1993, Dierauf and Gulland 2001). AGs are key agents in a veterinarian's armamentarium to quickly and effectively treat life-threatening infections in these debilitated animals.

The most common side effects of AGs are nephrotoxicity and ototoxicity. AGs are metabolized almost exclusively by glomerular filtration and injure proximal renal tubules. Nephrotoxicity is reversible and risk can be greatly reduced by kidney function and therapeutic monitoring, as well as maintaining hydration and avoiding other potential nephrotoxins (Jacobson *et al.* 1995).

Ototoxicity is frequently permanent because AGs injure cochlear hair cells, which cannot regenerate. AG ototoxicity initially presents as high frequency hearing loss and affects lower frequencies as damage progresses (Karasawa and Steyger 2011). Prevalence of ototoxicity ranges widely in published studies; 2% to 67% due to the many variables that can affect reported clinical outcome (Feldman *et al.* 2007, Huth *et al.* 2011).

There is a pronounced need for proactive strategies to avoid hearing loss because once initial signs of ototoxicity present, there are limited treatment options available to reverse or prevent further damage. Hearing loss can present during treatment but more often only presents after treatment cessation – even up to several weeks afterwards. Hearing loss often continues to decline after initial presentation for an additional several weeks and sometimes months (Fausti *et al.* 1984, Feldman *et al.* 2007). Hearing loss from AGs and its unique clinical presentation timeline present a need for preventive measures during treatment to reduce ototoxic risk.

Odontocete hearing loss can impede their foraging, navigation (Au 2002, André *et al.* 2003) and communication abilities (Schlundt *et al.* 2011). Wright (2011) tested the

foraging abilities of a deaf Atlantic bottlenose dolphin (*Tursiops truncatus*) compared to two healthy individuals of the same species and found the success rates of the deaf odontocete were significantly lower ($P < 0.0001$).

AG ototoxicity tends to affect high frequency hearing most. This may render odontocetes more vulnerable because it is an important part of their hearing range. Strategies exist for reducing or preventing ototoxic side effects - some of these may be appropriate for cetaceans.

In this paper we explore AG pathophysiology and strategies for reducing AG ototoxicity. Section 2 discusses the AG cytotoxic mechanism and explores its clinical manifestations. It reviews available literature regarding risk factors that increase susceptibility to AG ototoxicity. Section 3 reviews the potential effects of AG-induced hearing loss and the importance of hearing to cetacean functionality. Section 3 then discusses some clinical factors involved regarding the health of live-stranded odontocetes and how they could affect their susceptibility to AG side effects. Section 4 explores solutions that have been tried in other species and cetaceans, and identifies which solutions have the most potential to attenuate AG ototoxicity in live-stranded cetaceans in rehabilitation.

Section 2: Background on Aminoglycosides

2.0 Uses and Effectiveness

Aminoglycosides (AGs) are broad-spectrum antibiotics used in human and veterinary medicine for treatment of Gram-negative and Gram-positive bacterial infections, especially those involving virulent and resistant strains. Not only are AGs useful in treatment of identified infections, they are also well-suited to be used in combination with other antibiotics as initial empirical therapy, before culture and sensitivity results are available (Pagkalis *et al.* 2011). They are bactericidal and therefore act both more quickly and effectively than bacteriostatic antibiotics (Matt *et al.* 2012). In fact, using bacteriostatic medications to treat certain Gram-negative infections can promote endotoxemia (Dierauf and Gulland 2001). Additionally, bacterial resistance to AGs is less common than to other drug classes in humans (Avent *et al.* 2011) and some marine mammal populations (Greig *et al*. 2007, Schaefer *et al.* 2009). Allergic-type reactions to AGs, also, are uncommon (Arya 2007).

AGs are used in cetacean medicine, especially in live-stranded cetacean rehabilitation as these animals are often in critical condition and require swift, effective and broad-spectrum treatment to save their lives. AGs are most commonly administered parenterally, which is of particular benefit in ill animals where gastrointestinal absorption may be quite variable. This method of delivery prevents drug levels from varying due to digestive upsets (i.e. regurgitation, vomiting, or diarrhea) (Dierauf and Gulland 2001).

2.1 Pathophysiology

Although AGs have high clinical efficacy, their therapeutic window is very narrow because they carry a high risk for toxicity. Sections 2.1 and 2.2 provide a brief overview of select AG cytotoxic mechanisms and AG kinetics mechanisms and then explore characteristics of AG pathology in the ear. Section 2.3 continues with a discussion of clinical manifestations, applications and risk factors.

2.1a Bactericidal mechanism

AGs kill bacteria quickly by binding to the 30S ribosomal RNA subunit in bacterial ribosomes, which halts functional protein production (Rizzi and Hirose 2007). Most eukaryotic cell types are immune to such toxicity, but a few are not, such as those in cochlear tissues and proximal renal tubules (Arya 2007).

2.1b Ototoxicity

All toxicities associated with AGs are reversible except those affecting the inner ear because hair cells cannot regenerate (Schacht 1993, Selimoglu 2007). Ototoxicity presents as sensorineural hearing loss. High frequency hearing is affected first and most commonly. Hair cell destruction begins at the basal end of the cochlea, which transduces high frequencies. As damage travels towards the apical end, lower frequency sound reception is also compromised (Fausti *et al.* 1984). Ototoxicity subsequently also spreads to adjacent and supporting structures including the stria vascularis, spiral ligament, afferent nerve fibers and the eighth cranial nerve (auditory nerve) (Ryback 1986).

2.1b - 1 Molecular pathology

Upon entry into cochlear hair cells, AGs initiate apoptotic and necrotic cascades. AGs are highly polar, cationic (Matz 1993) and therefore basic at physiological pHs (Kukanich *et al.* 2004). Due to these characteristics, AGs disturb redox balances of cells and enzymes and also antagonize key reagents. AGs cause plasma membrane, lysosomal, and mitochondrial dysfunction, alter cell homeostasis by interfering with calcium, magnesium and potassium ion gradients (Takada and Schacht 1982, Li and Steyger 2009, Karasawa and Steyger 2011, Leitner *et al.* 2011, Xie *et al.* 2011) and cause epigenetic changes within tissues (Chen *et al.* 2009). By-products of these reactions are reactive oxygen species (ROS), or free radicals, that over-oxidize proteins, lipids and nucleic acids (Arya 2007). Many of these effects can also occur when AGs interact with proximal renal tubules (Jacobson *et al.* 1995). Dozens of pathways have been identified that describe these and other cochlear specific mechanisms in detail (suppl. note - 1). The relevant mechanisms that help explain clinical effects of AG ototoxicity are discussed in the following section.

2.1b - 2 Delayed onset of ototoxicity symptoms

Hearing loss can present days to weeks after cessation of treatment and may continue to worsen for an extended period afterwards (Forge and Schacht 2000). Several explanations for this phenomenon have been proposed that occur within the hair cell. These include initial compensation for damage from free radical oxidation as well as lysosomal sequestration and accumulation of AGs. Clinical manifestations of these mechanisms are discussed in section 2.3 and other sections thereafter.

Once AGs enter hair cells, they are sequestered by lysosomes within the cell. AGs accumulate, causing the size of the lysosome to increase substantially in size (Rizzi and Hirose 2007). Hashino *et al.* (2000) show that lysosomes can acquire extensive amounts of the drug, and likely for an extended period (suppl. note - 2) but eventually rupture. This triggers necrotic cascades and further increases the concentration of AGs in hair cell cytosol (Hashino *et al.* 2000).

Under normal physiological conditions, metabolic and enzymatic processes produce free radicals, or reactive oxygen species (ROS). Healthy animals have compensatory mechanisms that can combat damage from free radical oxidation whereby cell death from over-oxidation by ROS is mediated by cellular antioxidant systems. AGs interact with hair cell components to trigger additional and often excessive free radical formation. Cell homeostasis is initially maintained by up-regulation of antioxidant pathways but eventually the cell may be unable to compensate for the additional oxidative stress from AG exposure. Subsequently, cells initiate apoptotic and necrotic processes and pathways. Eventual inability to offset free radical oxidation from AGs could contribute to the delayed onset of AG-induced hearing loss (Xie *et al.* 2011).

The degree to which an animal can compensate for oxidative stress from AGs is reduced if the animal is in poor health and already under additional oxidative stress from other inflammatory conditions (Lautermann *et al.* 1995, Davies 2000). The implications of this in live-stranded cetacean rehabilitation are explored in later sections.

2.1c Nephrotoxicity

AG nephrotoxicity results in injury to proximal renal tubules and manifests as

non-oliguric acute renal failure. Elevated serum creatinine levels, decreases in renal blood flow and glomerular hydraulic conductivity and issues secondary to tubular injury such as hypokalemia and hypocalcemia are characteristic of AG nephrotoxicity. Elevated creatinine levels are also a known potential side effect of AGs in marine mammals (Dierauf and Gulland 2001). Proximal tubules can regenerate; therefore, depending on the extent of the damage, kidney function usually recovers (Jacobson *et al.* 1995) if medication is discontinued at first signs of impairment (Fausti *et al.* 1992). Normally, kidneys may take three to four weeks to recover their baseline function but kidneys with antecedent insufficiencies may take longer to recover, if at all (Jacobson *et al.* 1995).

2.2 Clinical Presentation of Nephrotoxicity and Ototoxicity and its Relationships to Aminoglycoside Kinetics

The relationships among nephrotoxicity, ototoxicity, aminoglycoside (AG) serum levels and AG clearance are complex and still not completely understood. However, several trends have emerged that clarify AG pharmacokinetics and potentially corresponding toxicity risks. Excessive serum peaks and shallow troughs increase both ototoxic and nephrotoxic risk, but even maintaining serum levels within the recommended range does not preclude either type of toxicity (Jacobson *et al.* 1995). Delayed AG clearance from decreased kidney function, whether preexisting or (Jacobson *et al.* 1995) iatrogenic, causes shallow trough levels, which allow more time for AGs to enter cochlear lymphatic channels and potentially damage hearing. This increases overall AG accumulation and additionally increases ototoxic risk (Fisman and Kaye 2000, Rizzi

and Hirose 2007). However, the absence of nephrotoxicity does not preclude the development of ototoxicity (Jacobson *et al.* 1995).

Monitoring AG serum levels to ensure that appropriate levels are maintained minimizes exposure of the kidneys to AGs and thus helps prevent nephrotoxicity (Mathews and Bailie 1987). Serum monitoring is less effective for the prevention of ototoxicity because AG serum levels are not good indicators that AGs have been cleared from inner ear tissues. AG uptake and clearance kinetics of AGs by kidneys compared to cochleae are mechanistically different. AGs have a protracted residence time in cochlear fluids (i.e. perilymph and endolymph). Cochlear kinetics have a distinct and possibly, a more convoluted relationship with serum levels compared to renal kinetics (Salt 2005, Arya 2007).

Blood serum half-lives of AGs in human patients with healthy kidneys range from 2 to 4 hours (Xie *et al.* 2011), however, AG half-life in cochlear lymph may exceed 30 days (Arya 2007). AG cochlear half-life has cumulative characteristics and increases with treatment duration, total dose (Huy *et al.* 1986) (suppl. note - 3) and other risk factors such as consecutive AG courses within a certain period (Mathews and Bailie 1987).

AG concentrations in cochlear fluids remain lower than blood serum levels throughout AG treatment (Arya 2007). However, although AGs are readily cleared from serum, AGs persist in these cochlear fluid compartments for extended periods. AGs enter hair cells from these fluid compartments (suppl. note - 4). Thus, AG concentration in hair cells continues to increase after cessation of treatment because AGs are able to enter hair cells long after treatment stops (Huy *et al.* 1986, Dulon *et al.* 1993, Salt 2005, Croes *et al.* 2012) showed that AG cochlear elimination half-life is biphasic, the first phase faster

than the second. In guinea pigs treated with a non-ototoxic course of gentamicin, the shorter half-life was two days while the long half-life was 5 to 6 months. Traces of gentamicin were found in cochleae up to 11 months after cessation of treatment (Dulon *et al.* 1993). AGs enter hair cells from cochlear lymph long after treatment cessation because AGs are not cleared from that compartment for weeks or months. Thus, if ototoxicity does present, hearing is likely to continue worsening long after treatment ends.

2.3 Ototoxicity in a Clinical Setting

Section 2.1b discussed potential biochemical mechanisms for the delayed clinical presentation of hearing loss such as initial compensation for reactive oxygen species (ROS) and lysosomal sequestration of AGs. Section 2.2 showed that hearing loss often continues to decline after initial presentation because of the protracted exposure of cochlear hair cells to AGs. This next section, 2.3a, explores results of laboratory and clinical trials to clarify timing of these events. Section 2.3b discusses how often ototoxicity occurs and will explain possible reasons for the wide variation of these results including study methods and population variability. Section 2.3c identifies risk factors that can increase patient susceptibility to ototoxicity.

2.3a Hearing loss: time to clinical presentation and to hearing loss plateau

2.3a - 1 Time to clinical presentation

The first signs of hearing loss present days to weeks after the start of treatment; often only after treatment has finished. Time of initial hearing loss presentation in

humans is observed to be as early as during treatment (6 - 14 days) and up to five weeks afterwards Hearing loss most commonly presents before the end of treatment with the presence of other ototoxicity risk factors and potentially in treatments lasting longer than ten days (Fausti *et al.* 1992, Raz *et al.* 1995, Black *et al.* 2004, Sha *et al.* 2006, Feldman *et al.* 2007, Tokgoz *et al.* 2011).

2.3a - 2 Time to hearing loss plateau

Once signs of hearing loss manifest, the degree of hearing loss may continue to worsen and only plateau weeks or months after treatment because the elimination half-life of AGs in cochlear fluids is both protracted and has cumulative characteristics (Li and Steyger 2009). Prolonged treatment leads to hearing loss in all patients (Xie *et al.* 2011). In humans, hearing has been seen to worsen several weeks after initial presentation (Feldman *et al.* 2007, Tokgoz *et al.* 2011) and up to four months in an extreme case (Duggal and Sarkar 2007). One of the few studies in rodents that continued to test hearing after treatment found hearing loss plateaued five weeks after treatment ceased (Pagkalis *et al.* 2011). Sha *et al.* (2006) and Chen *et al.* (2007) assessed hearing of patients that had been on AGs for no longer than seven days and saw that hearing did not worsen for longer than five weeks post treatment.

2.3b Ototoxicity prevalence and confounding factors

Results from human clinical trials show the prevalence of AG-induced ototoxicity is extremely variable. Studies report that 2% to 62% of patients experience ototoxicity. This is partly because ototoxicity manifests inconsistently between patients. However,

variability in reported prevalence can also be attributed to differences in experimental methods as well as study population. The previous two sections showed that the timing of hearing decline varies widely as well and most likely for the same reasons.

2.3b - 1 Differences in experimental methods: measurement and timing protocols

Hearing loss in patients can be underestimated for a variety of reasons including whether high frequency audiometry was utilized. Although hair cells that transduce higher frequencies are more vulnerable to ototoxicity, most studies only tested the lower half of the human hearing range (< 8000 Hz) (Schacht *et al.* 2012). If high frequency audiometry was not used, ototoxicity was often only detected once hearing loss extended below frequencies of normal speech (Rizzi and Hirose 2007). Studies that tested higher frequency hearing ranges (8000 Hz to 18000 Hz) in humans, found that ototoxicity was more prevalent (Fausti *et al.* 1993, Bailey *et al.* 1997, Feldman *et al.* 2007, Tokgoz *et al.* 2011, Harris *et al.* 2012). Studies that tested the entire human hearing range may represent more accurately AG ototoxicity prevalence in odontocetes, especially if cetaceans are as susceptible to ototoxicity as other mammals because research shows that the high frequency hearing range is vital to cetacean functioning (Finneran *et al.* 2005b).

Additionally, the testing method used can influence the sample population and create a sample bias. Various types of testing methods are used for testing hearing. Conventionally used, behavioral response hearing tests, require a patient to be alert and verbally or physically responsive to indicate they have heard the test sound. This generally excludes very ill patients from the testing population. Even if an ill patient feels well enough to undergo a hearing test, they often do not produce results that are reliable

enough to use in the data set (Fausti *et al.* 1993, Feldman *et al.* 2007, Forge and Schacht 2012).

Furthermore, some authors postulate that commonly used puretone audiometry is not sensitive enough to reliably detect high frequency hearing loss (Guthrie *et al.* 2008). Electrophysiological testing methods that test auditory brainstem response (ABR) and auditory evoked potentials do not require a physical response. They measure the subject's neural activity in response to a sound stimulus (Fausti *et al.* 1984, Schlundt *et al.* 2011). Few human studies used these electrophysiological methods. Type of hearing test used influences sensitivity to hearing loss and population accessibility, which can skew reported experimental results

The length of time after treatment that patient hearing was monitored can also affect reported ototoxic prevalence. Hearing loss may only present several weeks after treatment cessation and may continue to decline after treatment cessation. Studies that report lower ototoxic prevalence and milder degrees of hearing loss often lack long term follow up of hearing tests (Tange *et al.* 1995, Schacht *et al.* 2012). Conducting tests until hearing is stable can produce a more full assessment of the overall extent of AG induced hearing loss from a specific treatment course. In humans this time period may be as long as weeks to months after treatment finishes.

Most studies report ototoxicity occurred in 2% to 25% of cases (Huth *et al.* 2012). However, other studies that both used high frequency audiometry and tested patient hearing four weeks or more after treatment found prevalence to be much higher: between 47% and 63% (Feldman *et al.* 2007, Tokgoz *et al.* 2011, Harris *et al.* 2012). It is not always possible for studies to use high frequency audiometry and to conduct long-term

follow up. These tests can be cost-prohibitive and limited by patient cooperatively (Black *et al.* 2004, Harris *et al.* 2012).

Another confounding factor regarding ototoxicity reports is the definition of ototoxicity the researchers chose to use. Often the definition of ototoxicity was not consistent between studies (Schacht *et al.* 2012).

2.3b - 2 Variable risks between and within study populations: patient condition and prescribed treatment

Even if testing and reporting methods were identical between studies, a variety of clinical parameters can contribute to a patient's susceptibility to ototoxicity. Variables were not standardized across most studies, which could also account for the wide variation in reported ototoxicity prevalence. Often it is difficult to conduct studies that are standardized even if the study contains stringent exclusion parameters. This is because many variables such as individual patient health and prescribed treatment regimen are typically out of a researcher's control. Each case is unique regarding the patient's specific risk factors and the characteristics of the treatment that was prescribed (Fee 1980).

These clinical parameters include population health and treatment variability. Certain health conditions and ailments increase ototoxic risk in patients. Likewise, characteristics of the treatment a physician prescribes such as dose and duration can affect risk of ototoxicity (Avent *et al.* 2011, Schacht *et al.* 2012). The following section, section 2.3c goes into detail regarding these risk factors.

It is also important to note that reported values for ototoxic prevalence are likely conservative. Extremely sick and debilitated patients are at highest risk for ototoxicity,

but are often the most difficult population on which to conduct hearing tests. Due to the urgency of their condition, it is difficult to obtain baseline hearing information before AG treatment begins (Whelton *et al.* 1985). Additionally, often very ill hospitalized patients are unable to respond reliably to conventional behavioral hearing tests. Some studies use ABR methods, which do not require the patient to physically respond, to obtain hearing information. However this type of hearing test is cost-prohibitive and less commonly used in humans (Fausti *et al.* 2003). Additionally, very ill patients sometimes pass away before the end of treatment or before follow up tests can be conducted (Feldman *et al.* 2007). Therefore, the population that is most susceptible to ototoxicity is often omitted from reports in scientific literature.

Regardless of the wide variability in AG ototoxicity prevalence, at AG ototoxicity occurs often enough to warrant concern and a search for solutions.

2.3c Risk factors and some cetacean physiological analogs

The severity of cochlear damage and whether it occurs depends on a multitude of variables specific to each case. Many factors increase the risk of oto- and nephrotoxicity:

2.3c - 1 Poor health including inflammatory conditions other than renal insufficiency

Researchers postulate that ototoxicity likely manifests sooner, more severely, and more commonly if the patient is not in optimal health (Lautermann *et al.* 1995, Schacht *et al.* 2012). If human or animal patients are debilitated or already under oxidative stress, then their ability to compensate for the additional oxidative stress from AGs is likely compromised (Davies 2000). Rodent studies are usually conducted on healthy subjects

(Lautermann *et al.* 1985). Many human subjects did not have health problems other than the primary infection.

2.3c - 2 High area under the concentration-time curve (AUC): patient exposure and dosage frequency

The area under the concentration-time curve (AUC) is an integral that reflects patient exposure to a drug. AUC is based directly on AG serum concentration and treatment length. Higher AUCs are associated with a higher incidence of toxicity. AUC is influenced by the patient's AG clearance rate and by the dosage frequency; doses that are too frequent do not allow the patient enough time to clear the drug to achieve acceptable trough levels which increases AUC and toxicity risk (Croes *et al.* 2012).

2.3c - 3 Duration and dose

In humans, toxicity risk tends to increases after five days of treatment (Whelton *et al.* 1985, Avent *et al.* 2011) and substantially increases in treatments lasting more than ten days (Black *et al.* 2004, Rizzi and Hirose 2007). Among human infants who were exposed to AGs in the fetal stage, those who acquired hearing deficits had significantly longer mean treatment durations (15 days vs. 8 days, $P < 0.025$) (Pettigrew *et al.* 1988).

A very high total treatment dose increases the risk much more than high individual doses (Steyger 2005, Scaglione and Paraboni 2008,). However, if the dosing is too frequent and a low trough is not achieved then AGs can start to accumulate such that a high individual dose amount becomes more of a risk (Croes *et al.* 2012).

2.3c - 4 Consecutive AG treatments

AGs have a long residence time in the cochlea. Patients prescribed a subsequent course of AG treatment who were treated previously with AGs are likely to have at least a small amount of the drug in their inner ear from prior exposure. Giving a patient an AG treatment too soon after they finish a previous round of treatment increases risk for ototoxicity (Mathews and Bailey 1987). Most AG studies exclude patients if they have received AGs before the study. The time restrictions for excluding a patient because of prior AG exposure vary (e.g. Whelton *et al*. 1985, six weeks ; Sha *et al.* 2006, one month; Feldman *et al.* 2007, three months).

2.3c - 5 Renal insufficiency

Renal insufficiency increases ototoxic (Rizzi and Hirose 2007) and nephrotoxic risk (Jacobson *et al.* 1995) because delayed clearance leads to shallow troughs (lowest concentration of the drug in the blood before next dose is administered) and to greater accumulation of AGs in cochlear hair cells and renal proximal tubules. Ototoxic prevalence in patients with kidney problems was between 60% and 70% (Mathews and Bailie 1987, Feldman *et al.* 2007, Tokgoz *et al.* 2011).

2.3c - 6 Reduced kidney function in older humans and bottlenose dolphins

In humans, nephrotoxicity risk is higher in the elderly because kidney function declines with age (Nicolau *et al.* 1995). Elderly people also have higher risk of ototoxicity that may be due to a combination of reduced kidney function (Rizzi and Hirose 2007) and their predisposition to age-related hearing loss, known as presbycusis

(Fausti *et al.* 1984).

Similarly, risk of AG ototoxicity could be higher in older odontocetes because bottlenose dolphins experience age-related declines in both kidney function ($P < 0.0001$) (Venn-Watson *et al.* 2008) and hearing (Brill *et al.* 2001, Houser and Finneran 2006).

Glomerular filtration rate (GFR) is one metric used to measure kidney function in humans and bottlenose dolphins. Male bottlenose dolphins have both a lower GFR on average compared to females ($P < 0.0001$) (Venn-Watson *et al.* 2008) and are more susceptible to presbycusis compared to females ($P < 0.001$) (Houser and Finneran 2006). Male and aging bottlenose dolphins could be more susceptible to the ototoxic effects of AGs.

2.3c - 7 Dehydration

Hydration state influences renal kinetics, which directly affect AG clearance rate and corresponding toxicity risk. Dehydration can increase toxicity risk because it decreases renal blood flow and, depending on the extent, can profoundly suppress overall renal function in humans and other mammals including cetaceans (Dierauf and Gulland 2001, Papich 2012). Lecompte *et al.* (1981) found that dehydration in rats caused increased gentamicin levels in plasma and tissues, which leads to AG toxicity. Whelton (1985) and Bockenhauer *et al.* (2009) confirm that dehydration increases AG toxicity risk in humans. Dierauf and Gulland (2001) similarly caution the dangers of dehydration during AG treatment in marine mammals. This is especially important to consider in stranded cetacean rehabilitation and is discussed in more detail in section four.

However, although hydrating a patient can lower these toxicity risks, over-

hydration can cause other changes in AG kinetics. Overhydration decreased clinical efficacy and increased nephrotoxicity in rats. Thus, closely monitoring a patient is the safest way to ensure optimal hydration levels (Obatomi and Plummer 1993).

2.3c - 8 Diet

Fasting humans and rodents have a lower GFR. When fasting humans and rodents received AGs, they had a higher AUC and therefore likely a higher toxicity risk (Beauchamp and Labreque 2007, Pagkalis *et al.* 2012). Bottlenose dolphins have a lower GFR after fasting ($P < 0.0001$) (Venn-Watson *et al.* 2008) and as such, stranded and starving cetaceans could be more vulnerable to AG toxicity.

2.3c - 9 Circadian rhythm

GFR is regulated by circadian rhythm and is slower during the rest period. Slower clearance rates and larger AUCs have been seen in rodents and humans when dosed during their rest periods compared to their active periods. Dosing patients during their rest period can increase toxicity risk. Medicating subjects during their rest periods, showed significantly higher prevalence of nephrotoxicity in humans ($P < 0.004$) and in rats (Prins 1997).

In rats, administering medication during their rest period compared to during their active period increased ototoxicity prevalence (Soulban *et al.* 1990, Yonovitz and Fisch 1991 ($P < 0.05$)). Nephrotoxicity and high AUC decrease endo- and perilymph clearance overall (Croes *et al.* 2012) therefore dosing during the rest period could also increase ototoxic risk in humans and other mammals such as cetaceans.

2.3c - 10 Type of AG used and its relative cochlear toxicity

Each type of AG has a unique structural configuration. Presence and severity of side effects vary among different AG types. Reports vary, but Talaska and Schacht (as reported by Arya 2007) describe the order of roughly decreasing ototoxicity: neomycin, amikacin, gentamicin, tobramycin, netilmicin. Additionally, gentamicin has been described as more vestibulotoxic but less cochleotoxic than amikacin, neomycin and kanamycin (Rizzi and Hirose 2007). Herein we discuss AGs as a class and recognizes that the treating veterinarian or physician will be aware that the clinical outcome may be influenced by the type of AG used and will decide which is most suitable in each case.

2.3c - 11 Co-administered drugs

Certain drugs increase the risk of AG-induced oto- and nephrotoxicity. Nephrotoxic effects from AGs and cephalosporins are additive (Dierauf and Gulland 2001). Both the nephro- and ototoxic effects of vancomycin often act synergistically with AG toxicity (Tokgoz *et al.* 2011). AGs and the non-steroidal anti inflammatory, flunixin meglumide are also contraindicated in cetaceans due to increased nephrotoxic risk (Dierauf and Gulland 2001).

Administering loop diuretics such as furosemide concurrently with AGs increases risk of ototoxicity (Salt 2005) and nephrotoxicity (Dierauf and Gulland 2001). Loop diuretics likely increase the vascular permeability of the stria vascularis in the cochlea. The increased permeability allows AGs to move more quickly from the serum into the endolymph, which leads to earlier exposure of hair cells to AGs (refer to suppl. notes 4

for more on AG entry into the hair cell). Hearing loss can present after a few days in patients receiving both drugs, whereas a patient in a similar condition who only received AGs would experience hearing loss after 10 to 14 days (Salt 2005).

2.3c - 12 Noise exposure

Noise exposure and AGs synergistically damage hearing loss if the patient is exposed to noise before, during or after treatment. This synergistic effect can be seen in patients exposed to intense noise 30 days both before and after treatment. One of the mechanisms proposed for this effect is very similar to that of loop diuretics: increased vascular permeability between perilymph and endolymph compartments. Noise could also lead to faster uptake of AGs because it is likely to facilitate endocytotic uptake of AGs by outer hair cells and also enhances transduction through mechanoelectric transduction channels in inner hair cells (refer to suppl. notes 4 for more on AG entry into the hair cell) (Li and Steyger 2009).

2.3c - 13 Genetics

Some genetic mutations make certain individuals and populations more susceptible to AG ototoxicity, sometimes experiencing hearing loss after a single dose (Xie *et al.* 2011).

2.3c - 14 Fetal and neonatal age

AGs can cross the placental barrier and damage fetal hearing as well. Degree of sensitivity to AG ototoxicity may be affected by the age of the fetus because sensitivity

tends to be greater while hearing is in the critical phase of development (Xie *et al.* 2011).

If doses are too frequent, risk for nephrotoxicity and therefore presumably ototoxicity can be higher in neonates because their kidneys are less developed (Pacifini 2009).

Section 2 supplementary notes:

1. Additional cytotoxic mechanisms can be found in reviews by Karasawa and Steyger (2011) and Xie *et al.* (2011).
2. The lysosomes in the experiment ruptured after a few days, but Hashino *et al.* (2000) used a dose hundreds of times greater than that determined to be therapeutic. Hashino *et al.* postulate that lysosomes can go much longer before rupturing, possibly weeks or months.
3. Huy et al. (1986) found the shorter cochlear half-life was 13 hours (serum half-life 39 minutes) and the longer half life was 0.25 to 7.3 days for a single infusion of 5.4 mg. When the dose and duration increased, so did the half-lives, and after 30 days of treatment the AG cochlear half-life increased to 34.6 days.
4. AGs enter the inner ear through the bloodstream, although how they cross the blood-labyrinth barrier into perilymphatic scalae is uncertain. The fluid that resides in the perilymphatic scalae in the inner ear is called perilymph. It is postulated that AGs enter the cochlear duct either via ionic exchange pathways between the perilymphatic ducts or by diffusing through the stria vascularis (Salt 2005). The fluid in the cochlear duct is called endolymph. Two theories explain how AGs enter hair cells from endolymph: AGs enter hair cells through the stereocilia potentially by myosin mediated apical endocytosis or that AGs are driven from endolymph through stereocilial mechanoelectric transduction (MET) channels (Hashino 2000). For additional theories and uptake mechanisms see Salt 2005, Waguespack and Ricci 2005 as well as Xie *et al.* 2011.

Section 3: Odontocete Biology - Hearing, Stranding and Aminoglycoside Metabolism

3.1 Importance of Hearing to Cetaceans

Aminoglycosides (AGs) can cause permanent threshold shifts across all frequencies but primarily damage hair cells that transduce high frequencies. The evidence available suggests that odontocetes released with any type of hearing deficit have low survival rates (Wells *et al.* 2012), but it is extremely limited. High frequency hearing is important to odontocete survival on theoretical grounds because they rely on high frequency signals to echolocate (Au 2002) and potentially to communicate (Southall *et al.* 2008). However, it is not known how well they can adapt to high frequency hearing loss or which ecological functions could be lost in the event that an animal becomes impaired. Finneran *et al.* (2007) postulated that high frequency hearing loss can result in "concomitant reductions in temporal and spatial processing," which has significant potential to interfere with a wide range of odontocete survival functions to some degree.

Odontocetes produce high frequency sounds while foraging (Au *et al.* 2004), navigating, and communicating with conspecifics (Roch *et al.* 2007). Several species use high frequency sound (> 10 kHz) for communication. Dolphins regularly use broadband burst-pulsed calls ranging up to 150 kHz while communicating (Roch *et al.* 2007). The frequency of a beluga's whistles, significantly increased with depth ($P < 0.001$) (Ridgway *et al.* 2001). A high frequency hearing loss could interfere with an odontocete's ability to

communicate with conspecifics. Schlundt *et al.* (2011) postulate that a hearing impaired odontocete could be socially outcast.

Au *et al.* (1985) showed that a beluga changed its echolocation signals to adapt to ambient noise levels. In a noisier environment, the whale used higher frequency clicks for the same target detection task ($P = 0.01$). To compensate for a noise increase of about 15 dB, the beluga increased its click frequency by 60 kHz, producing clicks up to 100 to 120 kHz. High frequency hearing loss may impede odontocete capacity to adapt to high ambient noise levels (Ridgway and Carder 1997), an increasingly common problem caused by anthropogenic activities (Southall *et al.* 2008).

Research on captive odontocetes has shown some of the potential functional losses in animals with impaired high frequency hearing and suggested compensatory abilities. However, foraging success and other survival tasks have not been tested under free ranging conditions with animals that have known impairments, so potential survival adaptations and abilities of a wild odontocete with high frequency hearing loss are largely unknown.

Spectral properties of echolocation clicks and performance data from stationary target discrimination tasks are available from a captive false killer whale (*Pseudorca crassidens*) (Kloepper *et al.* 2010a, Kloepper *et al.* 2010b) and a bottlenose dolphin (Ibsen *et al.* 2007). These data were collected before and after the subjects experienced presbycusis, high frequency hearing loss caused by aging. The approximate peak hearing threshold of the false killer whale declined from approximately 100 kHz to 34 kHz and that of the bottlenose dolphin from 138 kHz to 40 kHz. Both the false killer whale and the bottlenose dolphin decreased the frequency and increased the intensity of their

echolocation signals to compensate for their losses.

Stationary target discrimination abilities of both animals were tested in some form before and after they experienced the hearing loss. Although test types and experimental methods were not identical, each study was consistent in its methods between baseline tests and those conducted after hearing loss. Kloepper *et al.* (2010b) saw a significant decrease in the false killer whale's task performance (36% reduction, $P < 0.001$) while Ibsen *et al.* (2007) found no significant reduction in function after acquiring a hearing deficit.

The results, that one animal displayed a functional loss while the other did not, are not inconsistent with theories about the adaptability of odontocetes. Presumably, compensatory ability and functional loss from a given degree of high frequency hearing loss is likely to vary among individuals and species. Turl *et al.* (1988) suggested that this variation could depend on behavioral adaptations and various types of processing abilities.

Differences between results may also be explained by differences in the targets used (Ibsen *et al.* 2007, Kloepper *et al.* 2010b). Task difficulty level, similarly, may not have been the same. The bottlenose dolphin discriminated between aluminum and brass targets. The false killer was asked to discriminate targets not based on the composition, but instead on the relative thickness of the target. It is possible that these two tasks require different types and levels of functioning. There are other potential differences between the studies that may further explain different results including degree of hearing loss, signal bandwidth, spectral shapes of clicks, and variation in auditory anatomy or physiology between the two species. Odontocetes change echolocation click time and

frequency parameters frequencies continuously to adjust to changing background noise or to acoustic characteristics of a target (Au 1993). High frequency hearing is needed for fine-focus spatial processing which may be helpful when pursuing small moving prey items (Roch *et al.* 2007, Au *et al.* 2009). The above studies tested captive animals with stationary targets. Whether the same degree of adaptability would be found in an odontocete pursuing a moving targets in the wild is unknown, however, Kloepper *et al.* (2010b) postulate from their study results that high frequency hearing likely helps with discrimination and range resolution of prey.

Elderly dolphins living with presbycusis in the wild have been found (Ridgway and Carder 1997, Li *et al.* 2013). Ridgway and Carder (1997) suggest that dolphins with high frequency hearing loss are able to compensate by following behavioral cues from other dolphins in their pod and by using their sight.

High-frequency cetaceans (Southall *et al.* 2008) may be particularly susceptible to effects of high-frequency impairment. Harbor porpoises use narrow-band high frequency clicks while echolocating, a behavior that might have evolved for hunting small prey close to the bottom, a highly-cluttered environment, and to prevent detection and predation by predatory odontocetes, such as killer whales. The upper limit of the hearing range in killer whales is not as high as harbor porpoises' (Szymanski et al. 1999, Morisaka and Connor 2007). In addition, harbor porpoises are vulnerable to aggression by other mid-frequency odontocetes (Pynn 2009). The ecologically significant role of high frequency hearing in wild harbor porpoises might result in lower chances of survival if released with high frequency hearing loss. If harbor porpoises were capable of lowering their click frequencies to match an impaired hearing range as other species of cetacean

study subjects were (i.e. Ibsen *et al.* 2007, Kloepper *et al.* 2010b), such an adjustment could increase their risk of predation.

Although specific information on the severity and type of functional losses that result from hearing impairment in odontocetes, have yet to be established, the available information suggests that the survival of rehabilitated odontocetes could be impeded if they were released with hearing deficits, including those in the high frequency range.

3.2 Known or Suspected Causes of Hearing Impairment from AGs in Odontocetes

There are many possible causes of hearing loss in odontocetes including AGs. Intense noise caused by anthropogenic activity can produce permanent and temporary threshold shifts (Finneran *et al.* 2005a). Presbycusis affects odontocetes as well; the prevalence may be linked to genetic vulnerabilities (Houser and Finneran 2006). Diseases (Szymanski *et al.* 1999) and parasites (Montie *et al.* 2011) can also cause hearing loss in odontocetes.

Although the available literature supports that AGs can cause ototoxicity in odontocetes, the potential prevalence and intensity of these effects have not been extensively explored. Finneran *et al*. (2005b) strongly implicated AG ototoxicity as the cause of hearing loss in a beluga. Several more recent case studies have suggested AG ototoxicity as a possible cause of hearing loss in other odontocete species (Houser and Finneran 2006, Pacini *et al*. 2010, Greenhow *et al*. 2011, Schlundt *et al.* 2011). Hearing tests are not commonly conducted on odontocetes. When hearing tests are conducted and

a hearing loss is identified, few studies can isolate AGs as the probable cause. Generally AGs cannot be excluded it as possible cause either (Ridgway and Carder 1997, Houser and Finneran 2006, Pacini *et al*. 2010, Greenhow *et al*. 2011, Schlundt *et al.* 2011). Reports by Finneran *et al.* (2005b) and Montie *et al.* 2011 are the only published studies to date containing baseline data. Finneran *et al.* isolated AG ototoxicity as a likely cause of hearing loss using the half-brother of the AG-treated whale as a baseline. Montie *et al.* (2011) ruled out hearing loss from one 9-day course of AGs by collecting audiograms before and after treatment. These results are discussed below.

Through 2013, the only published study that provides clear evidence for AG ototoxicity as a cause of hearing loss is Finneran *et al*. (2005b). They tested the hearing of two male half-sibling belugas. One had received amikacin to treat a virulent microbial infection. The two subjects had otherwise similar medical and life histories and were of similar age. The whale who did not receive AGs had normal hearing relative to the few samples of published beluga audiograms, with best sensitivity from 50 to 80 kHz, and functional hearing (thresholds < 120 dB *re* 1 μPa) to well over 100 kHz. The other beluga had high frequency hearing loss. His best sensitivity was from 30 to 35 kHz and he did not have functional hearing above 50 kHz (Finneran *et al*. 2005b).

The severity and persistence of the beluga's infection in the Finneran *et al.* study warranted a protracted treatment period. Although the course duration was longer than most live-stranded odontocetes in rehabilitation have received, the beluga in this study did not have additional health issues, which is not the case for stranded animals in rehabilitation. A stranded odontocete in poor health brings more clinical variables to the table that may increase their susceptibility to ototoxicity. Thus, there is a need for more

clinical evidence to evaluate the frequency and severity of AG-induced ototoxicity in stranded odontocetes.

3.3 Risk of AG Ototoxicity to Odontocetes

A review of the literature suggests, there are not enough published data to determine how sensitive stranded odontocetes are to ototoxic side effects of AGs. There are ototoxicity data from terrestrial mammals, however, that are sufficient to provide a baseline from which to investigate ototoxicity in odontocetes.

In terrestrial mammals, the outcomes of studies examining ototoxicity prevalence vary with experimental protocols and within and between species. Many case studies have speculatively attributed AGs as the cause of hearing loss in odontocetes (Houser and Finneran 2006, Pacini *et al*. 2010, Greenhow *et al*. 2011, Schlundt *et al.* 2011) and one reported five Risso's dolphins (*Grampus griseus*) that did not experience ototoxicity after being treated with AGs (Mann *et al.* 2010).

In humans and rodents, it can take weeks or months after treatment for hearing loss to present. The case could be the same for odontocetes. Thus, the report on Risso's dolphins (Mann *et al.* 2010) is not necessarily conclusive because the lag between treatment cessation and the hearing test may not have been sufficiently long enough to detect hearing loss.

The following sections discuss reasons why cetaceans may be at higher risk for ototoxicity and why this especially applies to those that have recently stranded.

3.3a Cetaceans may be more susceptible to ototoxicity due to reduced AG clearance

The renal system filters the blood and balances electrolytes and fluid volume. Ionic and water exchanges occur in the nephron before the filtrate is excreted (Jacobson *et al.* 1995). Marine mammals have hyper-functioning kidneys to adapt to a hypertonic environment. They can excrete very concentrated urine, allowing them to retain water and stay adequately hydrated. Their kidneys are reniculate (multi-lobulated). In odontocetes, each of these 300 to 1000 reniculi behaves like a functional kidney (Berta *et al.* 2005).

In the nephron, the glomerulus initially filters the blood before it reaches the tubules, which exchange ions and water to maintain homeostasis. The glomerular filtration rate (GFR) is a key indicator of kidney function. Clearance of creatinine, a waste product of muscle metabolism clearance, correlates closely with GFR. A decrease in GFR and elevation of creatinine serum levels are indicative of renal insufficiency (Jacobson *et al.* 1995).

The average human GFR (scaled to body surface area for a 70 kg human) generally falls between 89 and 122 ml/min/1.73m² (Jacobson *et al.* 1995). There are some data on GFR in small to mid-sized odontocetes. The mean GFR for bottlenose dolphins, normalized for surface area, is 188 ml/min/2.78m². The range is 95 (32-year-old male dolphin in renal failure) to 395 ml/min/2.78m² (Venn-Watson *et al.* 2008). As of 2013, GFRs of larger odontocetes are not published. Bottlenose dolphins are the only species of cetacean for which GFR information is available (Ridgway 1972, Venn-Watson *et al.* 2008). In terrestrial mammals, AGs are cleared almost exclusively through glomerular filtration. GFR values are therefore very closely related to AG clearance (Jacobson *et al.* 1995). Consistent with this explanation, AG half-life linearly decreases as GFR increases

in terrestrial mammals (Kukanich *et al.* 2004).

Compared to the terrestrial mammal relationship between GFR and AG half-life, Kukanich *et al.* (2004) found lower amikacin clearance rates and longer clearance times in a killer whale (*Orcinus orca*) and a beluga. Humans with a GFR of 96 ml/min/1.73m² clear amikacin at a rate of 1.3 ml/min/kg with a half-life of 2.3 hours. The beluga and killer whale clearance rates were much slower: 0.53 and 0.61 ml/min/kg respectively. The half-lives were 5.03 and 5.99 hours respectively. Assuming that the GFRs of the two large odontocetes in the study are similar enough to those reported for bottlenose dolphins to be comparable, the study subjects' amikacin clearance rates do not show the same relationship with GFR seen for terrestrial mammals.

Daily dosing, the interval used in this study, is the most popular interval for the loading dose period in terrestrial mammals. Intervals are extended if drug serum levels are too high after the loading dose period or if serum creatinine is elevated. Kukanich *et al.* (2004) recommended a dosing interval longer than 24 hours to allow time for the drug to be cleared and for appropriate serum trough levels to be reached.

The discrepancy between slow amikacin clearance in the Kukanich *et al.* study and high odontocete GFR values measured in others, suggests further limiting factors on odontocete AG metabolism. If bottlenose dolphin GFR values are similar to those of large odontocetes, then clearance rates were slower than expected. Kukanich *et al.* (2004) show that AG half-life increases with mass ($r = 0.97$) in terrestrial mammals, so GFR values for the beluga and killer whale subjects could have been lower than those of bottlenose dolphins. However, even if large odontocete GFRs are lower those of bottlenose dolphins due to differences in mass, it is unlikely that the difference in mass

fully accounts for such a protracted clearance time. Study subjects' creatinine levels remained within normal limits throughout the study (Kukanich *et al.* 2004). Therefore it is unlikely that GFR was below normal for the beluga and killer whale subjects. Until further study is conducted on AG clearance in odontocetes, intervals should be calculated to be conservative (long) for the initial loading dose period, in live-stranded odontocetes. Conservatism regarding the interval calculation would be especially beneficial as live-stranded odontocetes may have other health issues limiting AG clearance.

3.3b Stranded animals more susceptible to ototoxicity and nephrotoxicity

Stranding can be a behavioral adaptation to protect cetaceans from drowning in the event of severe illness, injury, or debilitating starvation (Rake 2010). Stranding itself is also highly stressful. Therefore, stranded marine mammals often enter treatment debilitated, malnourished and dehydrated and these conditions could make them more vulnerable to AG ototoxicity. Debilitated animals are likely to have a decreased capacity to compensate for oxidative stress from AGs. Furthermore dehydration and reduced kidney function may lead to decreased AG elimination, also increasing the risk of AG toxicity. These and other secondary issues can make the health of a stranded marine mammal far worse than that of a lab animal or a human at the start of treatment as follows:

3.3b - 1 Decreased ability to compensate for AG-induced oxidative stress and other complications

Free radicals are by-products of normal physiological processes (Xie *et al.* 2011).

The immune system's inflammatory response uses oxidative processes to protect the body from foreign pathogens and heal damaged tissues (Dierauf and Gulland 2001). To prevent the inflammatory process from damaging the body, antioxidant systems increase production of free-radical scavengers. However, an overproduction of reactive oxygen species (ROS) can overwhelm this system (Xie *et al.* 2011).

The endogenous inflammatory response involves lysosomal activity and produces ROS and nitrogen free-radical intermediates (Dierauf and Gulland 2001). In addition to endogenous inflammatory mechanisms, AGs induce their own inflammatory response involving similar processes and producing more free radical intermediates. Furthermore, AG cytotoxicity mechanisms repress endogenous cell-rescue pathways (Xie *et al.* 2011). If a human patient has pre-existing inflammatory conditions, endogenous antioxidant systems often have little reserve left to adequately mediate additional ROS damage from AGs, which means that a patient's ability to combat additional oxidative stress from AGs is lower than normal. A patient already experiencing conditions that cause oxidative stress is more susceptible to AG ototoxicity (Feldman *et al.* 2007). Similar mechanisms can be expected in stranded marine mammals.

Trauma, heat stress (Chang *et al.* 2007, Leon 2007) and psychological stress (Dierauf and Gulland 2001, Fowler 2009), infection (Forge and Schacht 2000), and malnutrition (Leon 2007), all cause oxidative stress and are common conditions in live-stranded cetaceans. Trauma can be present as either a cause of stranding or a result of being beached (i.e. sunburn and windburn) (Fowler 2009).

Psychological stress and heat stress elicit immune system inflammatory responses (Leon 2007). Dierauf and Gulland (2001) summarized that "elevated levels of stress

response proteins, such as those with roles in cell oxidative response and 'active cell death' were found in stressed cetaceans." Both Leon (2007) and Dierauf and Gulland (2001) agree that heat stroke can invoke Systemic Inflammatory Response Syndrome (SIRS), which can sometimes lead to endotoxemia. Psychological and heat stress not only cause oxidative stress but can increase corticosteroid levels (Leon 2007), which leads to decreased wound healing and immune function (Fowler 2009).

Starvation is a potential cause of stranding. While malnutrition can be associated with increased oxidative stress (Feldman *et al.* 2007), low dietary protein (Lautermann *et al.* 1995) additionally decreases ability to compensate for oxidative stress. Lautermann *et al.* (1995) showed that guinea pigs lacked key antioxidants that help mediate oxidative stress when they received a diet low in protein. Therefore, malnutrition not only increases endogenous free-radical production, but also decreases the body's intrinsic ability to compensate for oxidative stress, thereby increasing an animal's vulnerability to oxidative stress from AGs in two ways.

3.3b - 2 Dehydration, malnutrition, and heat stress: effects on kidney function

Live-stranded odontocetes may have pre-existing kidney conditions upon stranding, but dehydration and heat stroke from the act of beaching and laying on the beach can further decrease kidney function (Dierauf and Gulland 2001). This, in turn, increases vulnerability to nephro- and ototoxic side effects (Rizzi and Hirose 2007).

Humans can shunt blood from internal organs including the kidneys, in order to dissipate heat through the skin. Thus renal and other system failures are not uncommon in heat stroke patients (Leon 2007). Cetaceans depend on such shunting mechanisms to shed

heat (Perrin et al. 2009), and would thus be vulnerable to such failures as well.

Dehydration can result from beaching but can also be a pre-existing issue upon stranding. A sick cetacean in the wild may be emaciated and starving, therefore, the animal's access to normal sources of hydration such as blubber and food are restricted (Perrin *et al.* 2009). Fasting and debilitated cetaceans tend to drink seawater, which can sometimes increase their relative free water deficit (Berta *et al.* 2005). Mariposa or "sea water drinking" can also cause electrolyte and osmolality imbalances (Ridgway and Venn-Watson 2010). Dierauf and Gulland (2001) recommend concurrent hydration with AGs for all marine mammals. This is especially important for treatment of live-stranded marine mammals, because they are more likely to be dehydrated or have antecedent renal insufficiencies.

In addition to dehydration, if a cetacean is anorectic during AG treatment, then their risk for nephro- and ototoxicity might be higher as well. Behrend *et al.* (1994) found beagles that received less dietary protein during AG treatment had a higher AUC and experienced more of a decline in creatinine clearance than a control group.

In summary, the health issues of odontocetes upon stranding may render them more vulnerable to both nephrotoxicity and ototoxicity. When treating stranded marine mammals, taking into account the whale's diminished ability to combat oxidative stress and potential for diminished kidney function could benefit clinical outcome.

3.4 Reasons to Test Hearing of Stranded Cetaceans

A number of researchers highly recommend testing the hearing of stranded cetaceans (André *et al.* 2003, Houser and Finneran 2006, Schlundt *et al*. 2011, Wells *et*

al. 2012). The following sections summarize the many benefits of doing so:

3.4a Evaluation of hearing impairment as a factor contributing to stranding

Testing hearing of stranded cetaceans at the start of rehabilitation would provide information about the correlations between hearing loss and tendency to strand.

When a hearing deficit is identified in a live-stranded and rehabilitated odontocete, the loss can either be associated with the stranding or the result of treatment. Because of the stressed condition of stranded animals, hearing tests historically have been conducted after rehabilitation. Often, a hearing test is only administered after the animal has been exposed to AGs and not before (Pacini *et al*. 2010, Greenhow *et al*. 2011, Schlundt et al. 2011, Wells *et al.* 2012). In these cases, hearing loss could neither be conclusively attributed as a cause of stranding nor attributed to AGs.

Very limited observations have been taken as evidence that hearing loss can cause stranding (Mann *et al.* 2010, Schlundt et al. 2011, Wells *et al.* 2012). At least one subject in these studies had been treated with AGs. Thus, it is difficult to know whether the samples in these studies were representative.

The equipment necessary for conducting tests rapidly and with limited interference to treatment is becoming more available (Finneran 2009). Rehabilitation facilities are starting to test hearing of stranded animals, but the practice is not standard or common.

3.4b Monitor hearing impairment during rehabilitation process

Evidence suggests that cetaceans can experience AG-induced ototoxicity, but

more data are needed to learn about how sensitive they are to AG ototoxicity and how contributing risk factors affect the clinical outcome. Additionally, administering hearing tests after completion of treatment could allow us to see whether hearing loss worsens after treatment, for how long, and whether these results are comparable to those of other terrestrial mammals. These types of investigations could contribute to establishing how long after treatment an animal's hearing would need to be evaluated to determine whether it is releasable. Ultimately knowing how AGs affect hearing of cetaceans in rehabilitation can contribute towards establishing ways to prevent AG induced ototoxicity.

3.4c Health assessment prior to return to sea

Wells *et al.* (2012) note that high release failure rate in odontocetes with hearing loss should preclude them from release and warrants conducting hearing tests on odontocetes at the start of rehabilitation and especially prior to release. The integrity of stranded and rehabilitated cetacean hearing is not yet commonly evaluated whether or not AGs were used (Schlundt *et al.* 2011). Requiring a cetacean to have normal hearing relative to audiograms of their species and as a part of standard pre-release criteria would further insure survival once released.

3.4d Expand database of cetacean audiograms to understand species and population variability

Testing odontocete hearing in free-ranging populations (i.e. Nachtigall *et al.* 2008) in aquaria (i.e. Finneran *et al.* 2005b) and in the case of stranding (Greenhow *et al*. 2010) would help establish species-specific patterns and variability (Houser and Finneran

2006). Certain hearing thresholds may be normal for a particular species but not for another (Ridgways and Carder 1997). Popov and Supin (2007) note that a sufficient number of individuals is needed to determine mean and inter-individual variation standard of normal hearing. Limited information is available regarding hearing capability for species such as long-finned and short-finned pilot whales (*Globicephala malas* and *Globicephala macrorhynchus*, respectively) (Greenhow *et al.* 2011). Both species were classified as Data-Deficient on the IUCN Redlist (IUCN 2013). Mass strandings (i.e. Greenhow *et al.* 2011) would be of particular interest for such tests, as they often include a large number of relatively healthy individuals.

Establishing whether an audiogram of a stranded cetacean is within normal ranges of population variability for its species could help identify a hearing deficit. A better understanding of hearing capabilities at a population level could help identify even mild hearing deficits with more certainty.

3.4e Know more about behavior of odontocetes with hearing loss in a rehabilitation setting

Noting the behavior of an individual with hearing loss may uncover behavioral patterns that are common in hearing-impaired odontocetes. Whales and dolphins commonly mask hearing loss when interacting with people (Ridgway and Carder 1997) but abnormal social interactions with conspecifics may be more apparent because their ability to communicate is compromised (Schlundt *et al.* 2011). Their acoustic behavior too, could be indicative of the integrity of their hearing – an odontocete who does not vocalize at all or one who does vocalize but presents abnormal acoustic characteristics

may have hearing loss (Ridgway and Carder 1997). If a whale's hearing is not tested upon arrival, taking note of abnormal interactions with peers or listening for abnormal or a lack of vocalizations could uncover signs of hearing loss early in the rehabilitation process.

Wells *et al.* (2012) present pre-release evaluation criteria that include a behavioral section. They recommend checking that the animal is interacting with conspecifics in a way that is normal for its species. Expanding this section to suggest monitoring for social or acoustic behaviors indicative of hearing loss could provide another evaluative tool to screen for hearing deficits. In the future, having additional screening guidelines could also be helpful in flagging animals for testing at facilities with limited access to audiometric equipment.

Section 4: Strategies for Reducing or Preventing Ototoxicity

Although avoiding AGs would prevent hearing loss and kidney damage altogether, it is not a practical solution because the life-saving properties of AGs often outweigh their associated toxicity risks. There are few alternatives to AGs in many cases and therefore it is appropriate to explore techniques that decrease AG toxicity risk. Certain therapeutic strategies and modifications can reduce or eliminate ototoxicity and nephrotoxicity in lab animals and humans. The following sections review these solutions, some of which have already been used in odontocete rehabilitation. Those solutions with the greatest potential to reduce AG toxicity in live-stranded odontocete rehabilitation will be emphasized.

The primary purpose of this paper is to explore techniques that decrease or eliminate ototoxicity. However, because nephrotoxicity prevention has been studied more extensively and because techniques that lower nephrotoxic risk often lower ototoxic risk, methods that prevent one or both toxicity types are included in this section.

4.1 Strategies for Reducing or Preventing Toxicity in Other Species

4.1a Strategies that decrease AG exposure and area under the concentration-time curve (AUC)

4.1a-1 Extended Interval Aminoglycoside Dosing (EIAD):

Extended Interval Aminoglycoside Dosing (EIAD) is often used as the dosing scheme of choice because it minimizes toxicity risk, and also maintains or increases

clinical cure rate compared to traditional dosing schemes (suppl. section notes - 1) (Bailey *et al.* 1997, Fisman and Kaye 2000, Black *et al*. 2004, Scaglione and Paraboni 2008, Croes *et al.* 2012).

4.1a - 1.1 Kinetics

EIAD uses higher doses at less frequent intervals (suppl. section notes - 2). Extended intervals between doses allow the drug more time to clear and lower serum trough levels to be reached. This decreases the amount of time the patient is exposed to drug levels above thresholds that can cause toxicity (Croes *et al.* 2012) (suppl. section notes - 3). Barclay *et al.* (1994) found that EIAD lowers nephro- and ototoxicity risk even when total daily dosage is exactly the same.

Another reason EIAD is recommended is because, although high individual doses cause high serum peaks and cochlear endolymph concentrations, they do not correlate as closely with toxicity as a high total treatment dose. Antibiotic courses with a high total treatment dose can have lower individual doses but can maintain serum levels above recommended trough levels for a longer period. Thus, higher total dose usually renders a higher AUC, a significant predictor for toxicity. Even if the AUC is the same, high individual doses cause less toxicity than high total dose due to cochlear and renal tubule kinetics. The amount of AG that enters hair cells and proximal tubules is largely dependent on time of exposure as opposed to peak serum concentration. One of the ways that drugs enter hair cells and renal tubules is through a rate-limited active transport mechanism, meaning that a longer drug exposure time will allow more of the drug to enter vulnerable tissues compared to a shorter period of time regardless of the

concentration to which the tissues are exposed to (Steyger 2005, Scaglione and Paraboni 2008).

4.1a - 1.2 Cytotoxicity

EIAD is likely to decrease both nephrotoxic and ototoxic risk in humans in a clinical setting based on the mechanisms discovered in a lab setting.

In a double blind, randomized control study, Rybak *et al.* (1999) confirmed that EIAD significantly reduces nephrotoxicity in human patients with healthy kidneys in a hospital setting ($P < 0.004$). Extending the dosing interval is even more effective in reducing nephrotoxic risk in patients with renal insufficiency. Additionally, EIAD extends the amount of time that the course can last before reaching nephrotoxic threshold in patients at high risk for nephrotoxicity (Li *et al*. 2010, Pagkalis *et al.* 2011).

Maglio *et al.* (2002) provided valuable insight regarding effects of dosage regimen, comparing nephrotoxicity to ototoxicity:

> The correlation between ototoxicity and dosage regimen has been less well studied, partly due to the inherent difficulty in measuring ototoxicity in the clinical setting. Although it is clear that accumulation of aminoglycosides in the inner ear leads to the auditory and vestibular manifestations of ototoxicity, variations in patient toxicity threshold, the impact of dose/dosing interval, and the lack of adequate baseline data contribute to the poor differentiation of this toxicity in the patient care arena. Similar to nephrotoxicity, ototoxicity results from accumulation of aminoglycoside due to slow elimination in these tissues... Although less ototoxicity data is available, the results we have seem to parallel the effects found in the kidneys. Less structural and functional evidence of cochlear injury occurred in guinea pigs that received once-daily aminoglycosides compared with the same dose of aminoglycoside given as multiple doses [19].

Current knowledge regarding the mechanism supports the idea that EIAD likely reduces ototoxicity even though only a handful clinical studies confirm it. Researchers

agree that there is still sufficient evidence to warrant switching to EIAD to lower ototoxicity but that further study is needed (Barclay *et al.* 1994, Raz *et al.* 1995, Scaglione and Paraboni 2008).

Animal studies show that EIAD prevents ototoxicity but there is less evidence suggesting that EIAD prevents ototoxicity in humans (Barclay *et al.* 1994, Maglio *et al.* 2002). Many human studies have produced results that trend towards favoring EIAD as less ototoxic. However, those that found a decrease often did not have results with enough statistical power to render the risk reduction statistically significant (Tulkens *et al.* 1991, Hatala *et al.* 1996, Bailey *et al.* 1997, Fisman and Kaye 2000, Scaglione and Paraboni 2008). However most studies, and especially those that found no difference, only conducted behavioral hearing tests and did not test high frequency hearing (Munckhof *et al.* 1996, Bailey *et al.* 1997, Rybak *et al.* 1999) nor did they test the patient longer than one week after treatment (Tange *et al.* 1995). It is important to note that all rodent studies used an electrophysiological method to test hearing (Huy *et al.* 1986) (refer to section 2.3b for testing methods). It is likely that these lab animal studies were able to more accurately detect differences in ototoxicity prevalence between dosing regimens, compared to most human studies.

Another reason that many of the published human studies do not show a difference in ototoxic prevalence between EIAD and traditional dosing schemes, is that EIAD may reduce ototoxicity risk more substantially for higher risk patients. Extended intervals are more helpful for reducing nephrotoxic prevalence in patient populations at higher risk for nephrotoxicity (Li *et al.* 2010). Few studies that examine whether EIAD reduces ototoxicity prevalence include high risk patient populations in their analysis. As

mentioned in section 2.3b, very ill patients, while more susceptible to AG ototoxicity, are most likely to be excluded from statistical analysis because they are unable to undergo a baseline hearing tests (Raz *et al.* 1995). However, if EIAD does provide additional reduction of ototoxicity in high-risk patients, then it is likely to be very useful in cetacean rehabilitation as these animals are often far more debilitated than the average human patient.

When high frequency audiometry was used (n = 38) to test human patients, ototoxicity was significantly reduced (TID vs. SID netilmicin) ($0.1 > P \geq 0.05$) (Tulkens 1991). Raz *et al.* (1995) showed a significant reduction despite only testing the lower frequency range ($P < 0.05$) (n = 100). The mathematical model produced by Croes *et al*. (2012) projected that there was a non-linear relationship between AG concentration and hair cell death. A small increase in total AG concentration in the organ of Corti (from just below 8 mg/L to just above; SID to TID respectively) could increase the percentage of hair cells killed from ~ 0.5% to ~ 4.5%. Making sure low trough levels are reached, allowing the drug time to clear using the EIAD scheme, likely protects hair cells.

4.1a - 1.3 Efficacy

While EIAD decreases time of exposure and increases peak concentration to minimize toxicity, these pharmacokinetic modifications also optimize bactericidal efficacy, making efficient use of each dose (Nicolau *et al.* 1995, Dierauf and Gulland 2001). Clinical and bactericidal efficacy increases with the peak concentration of medication given because AGs exhibit concentration-dependent bacterial killing more so than time-dependent killing (Fisman and Kaye 2000).

The extended time between doses allows for more complete clearance of the drug, which decreases the chance of bacterial adaptive resistance (Bailey *et al.* 1997). Antibacterial activity is maintained during times when AG concentration is low because AGs are known for having one of the longest post-antibiotics effects (Pagkalis *et al.* 2011). The extended interval between doses takes advantage of this long post-antibiotic effect (Dierauf and Gulland 2001).

Extended intervals and higher doses from using EIAD directly decrease toxicity risk by means of interacting with cytotoxicity kinetics. They indirectly decrease toxicity risk as well, because high peaks optimize bacterial killing, giving EIAD the potential to decrease either the total dose needed or the treatment time, which in turn decreases a patient's AG exposure overall. EIAD improves clinical cure rate in most cases. Less bacterial regrowth means patients will not need to undergo consecutive treatments as often. Evidence supports EIAD as an effective method to decrease toxicity risk and enhance clinical cure rate.

4.1a - 2 Minimizing duration and total dose

Intuitively the simplest ways to decrease the area under the concentration-time curve (AUC) are decreasing AG concentration, i.e. reducing the dose, and decreasing the time the patient is exposed to the drug. Studies have confirmed that decreasing dose and duration both independently decrease toxicity. Generally, AG toxicity risk is lower if treatment lasts less than ten days (Rizzi and Hirose 2007) and treatments lasting 5 to 7 days are most often recommended (Whelton *et al.* 1985, Scaglione and Paraboni 2008). AGs are excellent for empirical treatment but often after a brief treatment (suppl. section

notes - 5), culture and sensitivity results can present the physician or veterinarian with alternative non-ototoxic drug options (Tokgoz *et al.* 2011). Protracted treatment may be unavoidable for certain infection types as determined by the attending physician or veterinarian but, close monitoring to prevent toxicity is especially important in these cases (Li *et al.* 2010).

The lowest possible therapeutic AG dose is recommended to prevent toxicity. Minimizing total dose by manipulating dosage frequency and treatment length as well as taking care to prescribe GFR-appropriate dosage and frequency can be very effective in preventing toxicity (Fee 1980, Bockenhauer *et al*. 2009).

4.1a - 3 Individualized Therapeutic Monitoring

Individualized therapeutic monitoring is a useful tool for improving treatment outcomes for a variety of reasons (Scaglione and Paraboni 2008, Papich 2012). It involves monitoring the patient's AG serum levels and kidney function and then adjusting the dose and frequency accordingly. Adjusting the dose and frequency to the patient's individual pharmacokinetics optimizes clinical efficacy and prevents toxicity.

Monitoring the patient's kidney function has two main purposes: first, it aids in the selection of a treatment regimen that allows the patient's kidneys enough time to process the drug such that serum levels descend to the desired trough. Second, it allows the clinician to detect a decline in kidney function during treatment as a result of nephrotoxicity and to reduce the dose and frequency of drug administration. This prevents further accumulation and toxicity. Without appropriate adjustments, kidneys can become progressively less able to clear AGs and with each subsequent dose, more and

more of the drug can accumulate, accelerating toxicity development.

Proper dosage amount and intervals are determined by selecting a loading dose and monitoring drug levels such as serum troughs and peaks (suppl. section notes - 6). This not only helps guide the clinician to make sure the antibiotic is being cleared properly to avoid toxicity, but also, to make sure that the medication reaches a peak concentration of maximal efficacy (Mathews and Bailie 1987). Trough recommendations vary with each infection (Pagkalis et al. 2011) but sufficient time between doses to allow trough levels to be achieved minimizes toxicity and drug accumulation overall and utilizes AG's long post antibiotic effect (Maglio et al. 2002).

Peaks are monitored to make sure the drug levels are high enough to be clinically effective - levels that do not reach the desired peak are often attributed as the primary reason for clinical failure (Nicolau *et al.* 1995). Peak AG levels are recommended to reach 8 to 10 times the minimal inhibitory concentration (MIC) of that specific infection, but not higher than 12 times. Peaks greater than 12 times the MIC render diminishing returns because toxicity continues to increase but gains in bactericidal and clinical efficacy are marginal (Scaglione and Paraboni 2008). Toxicity is reduced and bactericidal effects are higher when the peak AG concentration to MIC ratio is optimized (Nicolau *et al.* 1995) – therapeutic monitoring is a widely used approach to achieve these goals during AG treatment.

4.1b Maximizing kidney function

Proximal tubules can undergo morphological changes soon after drug administration, before clinical signs of nephrotoxicity are detectable. Gross clinical signs

of reduced kidney function such as raised AG troughs and creatinine levels often only appear 7 to 10 days after injury, thus proactively preventing nephrotoxicity and close monitoring are important. Reducing dosage frequency and amount accordingly as soon as changes in renal function are detected is highly recommended (Jacobson *et al.* 1995).

4.1b - 1 Hydration

Stranded odontocetes are nearly always dehydrated. Maintaining adequate hydration levels throughout treatment helps maintain adequate kidney function and minimize toxicity risk in rats and humans (Whelton 1985, Guthrie 2008). Prehydration likely has similar benefits (Bockenhauer *et al.* 2009). Additionally, rehydration can partially prevent and reverse additional AG accumulation from dehydration (Lecompte *et al.* 1981). So even if the animal was not hydrated as apart of overall treatment at the beginning of treatment with AGs, starting supplementary hydration as soon as possible could still be helpful.

Patients' fluid levels and kinetics can vary extensively throughout treatment process as their condition changes. Monitoring fluid levels closely and ensuring GFR appropriate dosing is recommended as the most effective way to minimize risk due to fluid fluctuations (Bockenhauer *et al.* 2009).

4.1b - 2 Dosing during times of high glomerular filtration rate (GFR)

With a once per day or less frequent dosing scheme it is worth considering examining the relationship of glomerular filtration rate (GFR) with circadian rhythm and metabolism. Sensitivity to the time of day while administering AGs can reduce toxicity

risk. Dosing during the active period decreases nephro- and ototoxicity risk because GFR is higher (Prins *et al.* 1997). GFR is also higher after the patient receives a high protein meal, therefore dosing after a meal can also minimize toxicity risk. Beagles consuming a high protein diet before and during AG treatment had a higher GFR, lower AUC and smaller prevalence of nephrotoxicity (Behrend *et al.* 1994).

4.1c Minimizing oxidative stress

4.1c - 1 Dietary protein

Making sure the patient is well-nourished can reduce oto- and nephrotoxicity for another reason. In addition to the potential pharmacokinetic benefits of adequate dietary protein intake, it also preserves the patient's ability to combat stress from free radicals resulting from AG treatment. A study on guinea pigs showed that those on higher protein diets were better able to compensate for oxidative stress caused by AGs and experienced less ototoxicity (Lautermann *et al.* 1995).

4.1c - 2 Concurrently administering additive therapies

Certain agents have the potential to reduce ototoxicity when used concurrently with AGs. Although many compounds interfere with a variety of hair cell death pathways, currently compounds that primarily mitigate oxidative stress from free radicals have the most potential as clinically viable options (Schacht *et al.* 2012). Factors involved in evaluating the clinical applicability of these compounds include how well therapeutic efficacy translates from *in vitro* to *in vivo* trials, long-term side effects and tolerability, dose and duration needed to provide adequate protection from specific AG

treatment regimen as well as interspecies variations in side effects and efficacy. Many are being explored and new therapies may be on the horizon. A few promising examples are described here. Solutions that have potential for marine mammal use are discussed in section 4.3.

Currently, *N*-acetylcysteine (NAC) and aspirin have successfully protected hair cells in human trials. They both decreased ROS production, decreased cochlear hair cell apoptosis and served as mild chelating agents (Chen *et al.* 2007, Tokgoz *et al.* 2011).

Patients receiving AGs and who additionally received 3 g/day of aspirin for 14 days, experienced significantly less ototoxicity ($P = 0.013$) (Sha *et al.* 2006). However using aspirin at high doses for extended periods of time increases risk of gastric ulceration (Rizzi and Hirose 2007), renal impairment (Jacobson *et al.* 1995) and at least temporary threshold shifts in hearing (Forge and Schacht 2000) and therefore may be unsuitable for certain patient demographics in humans. Another study investigated whether using a smaller dose for a shorter duration (1.5 g/day for 7 days) still protected hair cells effectively. Although ototoxicity was still significantly less in the aspirin group compared to the control group ($P < 0.04$), relative hearing decline compared to baseline function was still significant for both groups alike ($P < 0.001$) (Behnoud *et al.* 2009). The dose of aspirin a patient can tolerate compared to the efficacy of the dose in attenuating ototoxicity will determine whether aspirin is suitable for use in a patient undergoing AG treatment.

N-acetylcysteine (NAC) is used to treat and prevent oxidative stress damage from diseases and treatments affecting the inner ear and many organs, including the kidneys (Feldman *et al*. 2007, Tokgoz *et al.* 2011). It significantly reduced AG induced

hearing loss in high risk patients including those on dialysis in end-stage renal disease, those experiencing other chronic inflammatory conditions, and also those concurrently receiving vancomycin or who had prior exposure to AGs ($P = 0.002$ and $P < 0.001$; Feldman *et al*. 2007 and Tokgoz *et al*. 2011, respectively). Feldman *et al*. (2007) note that not only does NAC reliably protect hair cells, it is easily accessible and has been in use long enough to be determined as safe and having minimal side effects. It may be especially suitable for patients under extreme varieties of oxidative stress because it has shown a pronounced affinity for mediating free radical damage.

Other additive therapies with antioxidant properties are being explored including D-methionine (Campbell *et al.* 2007), α-lipoic acid, and α-tocopherol (a vitamin E analog). These are already used as dietary supplements and treatments in humans (Rybak and Whitworth 2005).

Iron chelating agents prevent AGs from reacting with iron and producing free radicals. Deferoxamine is established as a chelating agent in human medicine and has been successful in rodent clinical trials but in itself poses risks of neurotoxicity and ototoxicity (Xie *et al.* 2011). Dihydroxybenzoate is another iron chelator. Although it has not yet been tested in human trials, it has been successful in attenuating ototoxicity in rodent trials (Sinswat *et al.* 2000, Wu *et al.* 2001).

Histone deacetylation (HDAC) inhibitors prevent epigenetic changes caused by AGs in mouse cochlear cell explants, and may be another potential solution (Chen *et al.* 2009). A variety of HDAC inhibitors are already approved for anti-carcinogenic use in humans and are being explored as an additive treatment of many other diseases with epigenetic and inflammatory components (Glaser 2007). In addition to having antioxidant

properties, lipoic acid and vitamin E metabolites also have HDAC inhibitory properties (Dashwood and Hob 2007).

Apramycin, a relatively newer synthetic aminoglycoside licensed for veterinary use, also attenuates AG-induced ototoxicity as it shows little reactivity towards eukaryotic mitochondrial ribosomes. This drug is currently licensed for veterinary use (Matt *et al.* 2012) and has been in use since the 1980s (Johnson 1997).

A single compound that can successfully interfere with each one of the vast array of ototoxic pathways and thus provide complete protection may not exist (Xie *et al*. 2011). However compounds that provide high degrees of protection from at least some pathways have the potential to ultimately increase AG tolerability. This could preserve patient hearing as well as increase bactericidal efficacy and clinical cure rates by increasing patients' toxicity thresholds (Karasawa and Steyger 2011).

For additional therapies under investigation see Rybak and Whitworth 2005, Huth *et al.* 2011, Leitner *et al.* 2011, and Bayindir *et al*. 2013.

4.1d Proactively testing hearing and ceasing treatment at first sign of ototoxicity

Although hearing loss sometimes only presents after the patient has completed a course of AGs, there is a consensus in the published literature that if hearing loss is found during treatment, immediately discontinuing AG treatment increases the chance of preserving the patient's remaining hearing. Hearing may continue to deteriorate after treatment cessation, however, it is much more likely to do so if treatment continues. Thus, monitoring the patient for hearing loss early and often is highly recommended (Black *et al.* 2004**)**. Based on the variability of time until clinical presentation of hearing

loss and in dose and duration of treatment, the American Academy of Audiology (2009) recommends monitoring human patient hearing biweekly or weekly during treatment and additionally for several months after drug discontinuation.

4.2 Solutions Applied to Cetaceans

Utilizing EIAD by dosing once daily is not a new treatment strategy in marine mammals to prevent nephrotoxicity (Dierauf and Gulland 2001). Kukanich *et al.* (2004) investigated the pharmacological values of this dosing strategy. They emphasize the importance of accurate inter-species scaling methods and the importance of therapeutic monitoring. They strongly suggest that even while administering dosage amounts within recommended ranges, the dose interval should be extended. See the above publication for details on how they scaled an appropriate dose and for their therapeutic monitoring techniques.

The report of note that used extended interval dosing is Montie *et al.* (2011). A live-stranded pygmy killer whale *(Feresa attenuata)* was given 21 mg/kg every 48 hours for 9 days, a dosage interval that Peritoneal Dialysis International (Li *et al.* 2010) recommends for patients with decreased AG clearance abilities. This course was administered to the animal twice within a several month period. A hearing test utilizing pure-tone high frequency audiometry was conducted before antibiotic administration and after treatment conclusion. Neither hearing test found evidence of high frequency hearing loss. The second hearing test was conducted more than a month after the first course but only five days after the second (Montie *et al.* 2011), a period too short to rule out future development of hearing loss from the second course. Risk of ototoxicity was likely higher

for that second course because consecutive AG courses increase ototoxicity risk.

Still, results strongly indicate that it is unlikely that the first course caused any ototoxicity. Although Montie *et al.* did not demonstrate conclusively that short courses of treatment or using EIAD were singularly or jointly responsible for preventing ototoxicity, their report shows it is possible for ototoxicity from AGs to be prevented and provides guidance about treatment methods that prospective studies can explore further.

Research in other mammals implicates that high total dose is a more common contributor to ototoxic risk than high individual doses (Rizzi and Hirose 2007). This case provides evidence that this trend could apply in odontocetes as well. Despite this being one of the highest doses given to an odontocete in the literature published to date, the whale did not experience ototoxicity, whereas all other cases that attribute AGs as a potential cause of ototoxicity reported the use of doses from up to 16 mg/kg more frequently than every 48 hours.

Regarding another toxicity-attenuating solution already tried in marine mammals, The CRC Handbook of Marine Mammal Medicine (Dierauf and Gulland 2001) recommends supplementary concurrent hydration with AG use to help prevent nephrotoxicity. They did not recommend concurrent hydration to prevent ototoxicity, but it is likely to have therapeutic benefits for this reason as well.

4.3 Discussion and Recommendations

The ultimate goal of this review has been to provide a foundation for recommendations that will enhance cetacean rehabilitation protocols. Exploring three main questions will help achieve this goal:

1. How susceptible are odontocetes to AG-induced ototoxicity?
2. What are the functional consequences of odontocete hearing loss, particularly at high frequencies?
3. What are some potential ways to prevent ototoxicity in odontocetes treated with AGs?

4.3a Which strategies have the most potential to be effective in improving stranded odontocete rehabilitation success rates?

Prospective studies will provide much needed information on AG toxicity and dosing, but there are other strategies that may be logistically more achievable in the short term. For example, it is likely that groups involved in cetacean rehabilitation, research and medical management already have useful unpublished data that, if shared, could further contribute to answering questions about AG ototoxicity and methods for reducing it. Retrospective studies that test the hearing of stranded cetaceans or of individuals that have received AGs in the past could provide valuable insight as well. Additionally, observational studies that test the functional abilities of hearing-impaired cetaceans, and studies that monitor AG clearance rates of cetaceans undergoing treatment could help narrow the scope of future studies. Each type of information collected and shared has the potential to provide a foundation from which scientifically-based protocol updates can be created. Ultimately, they can also involve groups in other countries with burgeoning cetacean rehabilitation and collection facilities. This would make them aware of the importance of auditory health to cetaceans and encourage mindfulness of AG-associated issues.

4.3a - 1 Conduct retrospective and observational-type case studies or publish existing data

There are a number of ways to potentially mitigate risk of AG ototoxicity to cetaceans. Literature from a range of species and study types demonstrate many possible techniques for risk management that could be explored with prospective studies. However, the dearth of baseline information available in published literature warrants attention. Therefore, before discussing new potential clinical techniques to explore, I will address the types of data that, if published, have the potential to help fill in gaps in the literature.

By far, the fastest and most efficient way to add to the body of knowledge on a topic, is to share knowledge and data that already exist. If data do not exist yet, retrospective, observational and case studies can provide a wealth of information more easily than designing and conducting an entirely new investigation. Publication of all such reports is invited and encouraged.

In order to further explore AG pharmacokinetics and metabolism in odontocetes, reports of AG clearance times, serum levels, and scaling methods are needed. This will encourage veterinarians and researchers to explore cetacean-appropriate dosage amount and frequency.

There are many options to learn more about odontocete susceptibility to AG ototoxicity, and the functional effects of treatment or hearing loss. I recommend conducting hearing tests on odontocetes that have received AG treatment in the past or publishing those already collected. If an odontocete received AGs during rehabilitation and was released with or without a hearing test, post-release tracking is encouraged as

well as publication of the outcome. If an odontocete strands, hearing loss should be investigated as a cause of stranding. Knowing how common hearing loss is amongst stranded animals will help understand the possible effects of hearing loss.

Testing the functional abilities of animals with high frequency hearing loss due to AG ototoxicity or presbycusis will help understand how well different species and individuals are able to compensate for hearing deficits of varying degrees. Similarly, observing interactions between a hearing-impaired individual with conspecifics and recording the animal's vocalizations may help distinguish signs of hearing loss and its consequences.

Sharing reports of all of these findings is encouraged.

4.3a - 2 Administer hearing tests in a rehabilitation setting

There is limited information regarding when hearing loss presents, how severe the effects can become in odontocetes and how long after treatment hearing decline stops. Therefore, I recommend administering a minimum of two hearing tests, the first at the start of rehabilitation and the second prior to release. However if the veterinarian deems that the animal can tolerate further hearing tests and if logistics allow, more hearing tests will help answer these questions more quickly and conclusively. For investigative purposes, I recommend testing the animal upon arrival at the facility, during as well as after treatment at increments and finally, before release. When more information is gathered, guidelines can be created that designate the minimum number of tests needed to provide an accurate assessment of the effects of AG ototoxicity on the rehabilitated animal. Until then, testing when possible to identify hearing loss is the best way to

prevent hearing impaired animals from being released.

4.3b Strategies involving clinical modifications

Here I discuss clinical modifications that might help minimize ototoxic risk in odontocetes.

4.3b - 1 Using AGs as empirical treatment then switching to less ototoxic drugs

Striving to use AGs (usually in combination with other drugs) only as empirical treatment unless culture and sensitivity results deem them absolutely necessary would limit exposure overall. Thus, treating with AGs for 2 to 5 days and then switching to a less ototoxic drug would help decrease ototoxic risk.

4.3b - 2 Utilizing EIAD and potentially extending the interval further

Extending the dosing interval would help in a number of ways. It has the potential to increase clinical outcome and to decrease cumulative AG exposure that causes ototoxicity and nephrotoxicity. Dierauf and Gulland (2001) recommend it for optimizing bacterial killing and for preventing nephrotoxicity in marine mammals. Based on cases published by Kukanich *et al.* (2004) and Montie *et al.* (2011), dosing intervals between 24 and 48 hours are likely to be appropriate but further investigation is needed.

4.3b - 3 Individualized therapeutic monitoring

Methods described by Kukanich *et al.* (2004) show efficient ways to monitor an odontocete's kidney function as well as AG serum levels and clearance rate. This helps

ensure clinical efficacy, decreasing the need for longer or subsequent consecutive treatments. It also helps the veterinarian determine a dosage amount and interval that is appropriate for the animal's clearance rate and helps prevent unnecessary AG accumulation from kidney function decline due to nephrotoxicity.

4.3b - 4 Dosing during the day and after a meal

An odontocete clearing AGs too slowly is likely more of a concern than an odontocete clearing an AG more quickly than desired. Therefore dosing when GFR is higher, during their active period and after a meal, is likely to minimize toxicity risk.

4.3b - 5 Hydration therapy

Dierauf and Gulland (2001) recommend concurrent hydration therapy while using AGs in marine mammals. Studies have shown that pre- and post-hydration helps as well. Hydration therapy likely helps prevent ototoxicity as well because by decreasing nephrotoxicity, it helps avoid toxic AG accumulation.

4.3b - 6 Additive therapies

Many additive therapies are being investigated to safely reduce ototoxic risk. So far the two therapies that have been investigated most extensively in humans are Aspirin and NAC. The effects and appropriate dosages of Aspirin are already established in whales and it is likely that Aspirin would attenuate hearing loss from ototoxicity to some degree in odontocetes. However, amount of medication and duration of treatment needed to significantly reduce ototoxicity is still in the early stages of investigation in humans and

likewise would need to be investigated in odontocetes. Aspirin and other non-steroidal anti-inflammatory drugs can cause declines in kidney function (Dierauf and Gulland 2001) and gastric bleeding in odontocetes (Barnett 2002). The risks of using Aspirin may outweigh its oto-protective benefits. The amount of oto-protection aspirin offers compared to the amount needed has yet to be established in odontocetes.

Although safety of NAC has not been investigated extensively in odontocetes, it has proven relatively safe for most if not all species for which clinical results are available. It is most helpful in preventing damage from AGs in patients undergoing oxidative stress, which odontocetes in rehabilitation invariably are. NAC may have more potential for use in marine mammal oto-protection than Aspirin.

Antioxidants enhance the ability of malnourished, debilitated animals and humans to combat oxidative stress. Therefore many of the antioxidants mentioned in 4.2c and its references including D-methionine, vitamin E and α-tocopherol, have the potential to be useful in odontocete oto-protection. Vitamin E decreased ototoxicity prevalence in rodent trials, but did not provide significant protection in humans (Kharkheli *et al.* 2007). Although the oto-protective potential of Vitamin E did not translate from rodents to humans, the human trial was fairly small and did not include high risk patients. Forge and Schacht (2000) note that antioxidants are likely to be more effective in debilitated and malnourished patients, therefore Vitamin E may still prove useful in attenuating ototoxicity in odontocetes should it be investigated in the future. Vitamin E has been safely used in cetaceans (Dierauf and Gulland 2001, Finneran *et al.* 2005b), which makes it easier to investigate as a potential solution.

A-tocopherol, a vitamin E analog, has shown significant results in preventing

ototoxicity in lab animals, more so than did Vitamin E itself and may be useful in odontocete rehabilitation in the future. It is used in humans as a dietary supplement but its safely has not been formally investigated in whales as of 2013.

4.4 How Might These Solutions be Tested?

Many of these solutions can be tested in a rehabilitation, research or aquarium setting, but some of these settings may facilitate certain tests.

Zoo and aquarium collections are in a good position to collect hearing tests on animals that have received AGs in the past. From these results, correlations between AG treatment and hearing loss can be investigated.

Prospective studies could be conducted on stranded animals. Dolphins and pilot whales have the highest tendency to strand. Focusing on these species would facilitate research efforts and sample size would grow quickly.

With more auditory and medical data gathered, we can start putting together an idea of how the hearing of odontocetes is affected by this class of drugs and can work together to revise treatment protocols, ultimately for the betterment of odontocete rehabilitation success rates and conservation efforts.

Section 4 supplementary notes:

1. However, smaller dosage intervals may be more effective if the patient is in a hyper/heamodynamic condition such as in early stages of sepsis or has an infection with a high rate of adaptive resistance such as *Pseudomonas aeruginosa* (Fisman and Kaye 2000).
2. I.e.16 mg/kg amikacin sid vs. 8 mg/kg amikacin bid
3. These thresholds are dictated by appropriate trough and MIC levels which vary between AG and infection type
4. Avent *et al.* (2011) suggest avoiding using AGs for longer than 48 hours for empirical treatment.
5. See Fisman and Kaye 2000 for peak and troughs level recommendations for specific infections and degrees renal function. See Avent *et al.* (2011) for therapeutic monitoring technique options for humans

and Papich 2012 for techniques in veterinary medicine

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