Altered Bilayer Elasticity as a Novel Mechanism for Aminoglycoside Antibiotics' Toxicity

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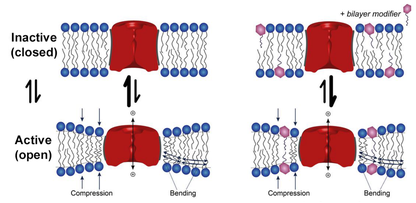
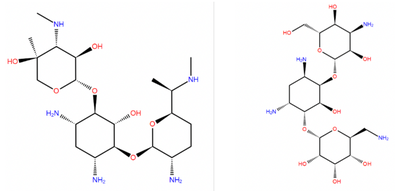
INTRODUCTION

The lipid bilayer regulates membrane protein function and assembles spontaneously due to the hydrophobic interactions between the non-polar tails. When the protein undergoes conformational changes, the lipid bilayer must accommodate by altering its elastic properties, composed of both the compression and bending moduli. to understand the effect that the drug has on the membrane, thus, correlating to the toxicity of the drug itself.



Andersen, in press

Ototoxicity is the pharmacological adverse reaction on the cochlea, the auditory nerve, or the vestibular nerve, characterized by cochlear or vestibular dysfunction (Ganesan et al, 2018). Of the aminoglycosides known to cause ototoxicity, Gentamicin and Kanamycin, although structurally different, they have the same surface potential. Gentamicin is the most commonly used aminoglycoside antibiotic used to treat gram-negative bacteria, but it known to cause vestibular toxicity, resulting in dizziness, ataxia (impaired balance or coordination), and/or nystagmus (involuntary eye movement). Kanamycin is mostly used against streptomycin-resistant bacteria, but can cause cochlear toxicity, resulting in permanent hearing loss.



Gentamicin **Kanamycin**

2D structures of Gentamicin and Kanamycin, respectively; images obtained using Canvas, Schrödinger, LLC, (New York, NY)

OBJECTIVES

- Identify whether changes in bilayer elasticity is a nonspecific mechanism for aminoglycoside antibiotic's toxicity
- Ascertain that aminoglycosides do not influence bilayer elasticity in neutral vesicles
- Investigate the role of aminoglycosides' charge in binding to lipid bilayers (of which have different charges)
- Ideally, this research would allow pharmaceutical companies to produce less toxic and more effective drugs before screening molecules for millions of dollars

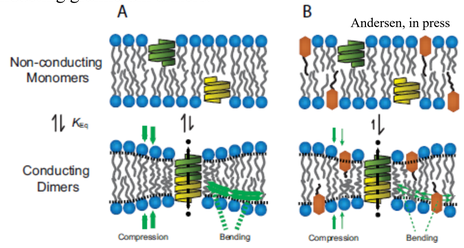
MATERIALS

The zwitterionic (net neutral) 1,2-Dierucoyl-*sn*-Glycero-3-Phosphocholine (DC22:1PC), and the anionic 1,2-Dierucoyl-*sn*-glycero-3-Phospho-L-serine (DC22:1PS), and 1,2-Dierucoyl-*sn*- Glycero-3-Phosphoglycerol (DC22:1PG), all in chloroform (25 mg/mL) were from Avanti Polar Lipids (Alabaster, AL). Methanol was from VWR (Radnor, PA). Thallium nitrate (TINO3), Sodium nitrate (NaNO3), HEPES, Gentamicin sulfate (Gen), Kanamycin sulfate (Kan), and gramicidin (gD) from *Bacillus aneurinolyticus* (*Bacillus brevis*) were from Sigma-Aldrich Co (St. Louis, MO). 8-Aminonaphthalene-1,3,6-Trisulfonic Acid, Disodium Salt (ANTS) was from Invitrogen (Eugene, OR). All materials were used as received; all stock solutions of buffers and quenchers were prepared before the experiment. Solutions were dissolved in deionized water, at pH 7 with Sodium hydroxide (NaOH) and Nitric acid (HNO3) and stored at 12.5 °C. ANTS solution was made with 25 mM ANTS, 100 mM NaNO3, 10 mM HEPES, and stored in the dark. Buffer was made with 140 mM NaOH and 10 mM HEPES; quencher was made with 50 mM TINO3 94 mM NaNO3 and 10 mM HEPES. 500 µg/mL of gD in powder form dissolved in methanol and was stored at -40 °C.

METHODS

GRAMICIDIN-BASED FLUORESCENCE ASSAY

Gramicidin (gD) is a 15 amino acid antibiotic peptide. When two gD monomers from opposing bilayer leaflets dimerize, they form a monovalent cation permeable channel. As the bilayer becomes more elastic, the monomer↔dimer equilibrium is shifted towards the formation of conducting gramicidin dimers.

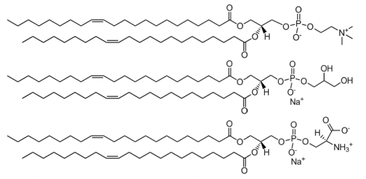


Andersen, in press

LARGE-UNILAMELLAR VESICLES (LUVs)

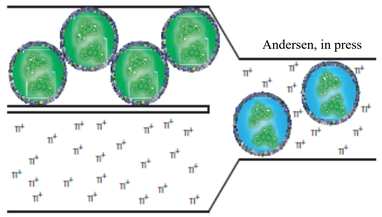
Large unilamellar vesicles (LUVs) were made using a three-part process of freeze-thaw cycles, extrusion, and elution. The LUVs are made of a glycerophospholipid(s) and gramicidin (gD), dried under nitrogen gas, and loaded with 8-aminophthalene-1,3,6-trisulfonic acid disodium salt (ANTS).

GLYCEROPHOSPHOLIPIDS:



STOPPED-FLOW SPECTROFLUOROMETRY

The fluorescence produced by ANTS is quenched by thallium ions (Tl+) very rapidly in gramicidin channels. The Tl+ influx rate reflects gramicidin activity and is a proxy for bilayer perturbation. The rate of fluorescence quenching was measured at 25 °C using a SX-20 stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, UK) in the single-mixing mode.



Each quench trace was analyzed separately using MATLAB R2019a (The MathWorks Inc.) and quench rates were determined by fitting to a Stern-Volmer modified stretched exponential (Peyear 2020).

$$F(t) = \frac{2.5 \cdot F(\infty,q) - F(0,q)}{1.5} + \frac{2.5}{1.5} \cdot \frac{F(0,q) - F(\infty,q)}{1 + 1.5 \cdot \left(1 - \exp\left\{1 - (1 + t/\tau_0)^{\beta}\right\}\right)}$$

I used these equations to calculate the quench rate and to quantify the drug-induced changes in the number of conducting gD channels (Peyear 2020), respectively.

$$Rate(0) = \frac{1}{[Tl^+]_e} \cdot \left.\frac{d[Tl^+]_i}{dt}\right|_0 = \frac{\beta}{\tau_0} \cdot (1 + t/\tau_0)^{\beta - 1} \cdot \exp\left\{\left(1 - (1 + t/\tau_0)^{\beta}\right)\right\}\Big|_0 = \frac{\beta}{\tau_0}$$

$$RelRate = \frac{Rate}{Rate_{cntrl}} = \frac{Rate_{drug}(0)}{Rate_{cntrl}(0)}$$

DYNAMIC LIGHT SCATTERING (DLS)

Vesicle size dispersity was measured by DLS using a Litesizer™ 500 (Anton Paar, Austria). By using the Brownian motion model and the Stokes-Einstein relation, both the hydrodynamic diameter and polydispersity index (PDI) were calculated (Koppel, 1972). The average LUV hydrodynamic diameter was 130 nm, and the polydispersity index (PDI) was 0.15, which has been associated with a monodisperse distribution of particles. Using the Smoluchowski model, mean zeta potential was also measured using the DLS (Kirby and Hasselbrink 2004).

RESULTS & DISCUSSION

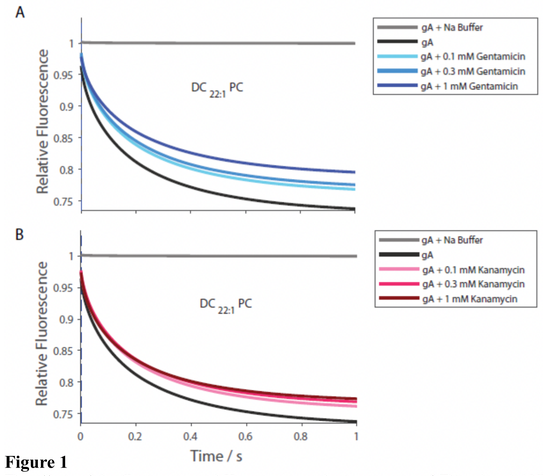


Figure 1

The effects of the Gentamicin and Kanamycin on the time course of Tl+-induced ANTS fluorescence quenching in $DC_{22:1}PC$ vesicles. The gray line represents the quench trace in the absence of amphiphiles or Tl+. The black curve represents the quenching of LUVs in the presence of Tl+ only; the colored curves denote results with increasing antibiotic concentrations.

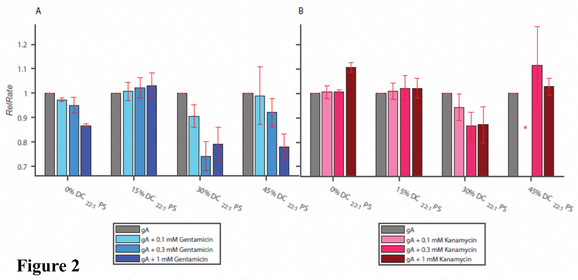


Figure 2

The relative quench rates observed with Gentamicin (A) and Kanamycin (B) with 0%, 15%, 30%, and 45% $DC_{22:1}PS$ lipids in the LUV membranes. *In panel B, there is no bar at 0.1 mM Kanamycin and 45% $DC_{22:1}PS$ because there were no quenchable vesicles, I do not understand why.

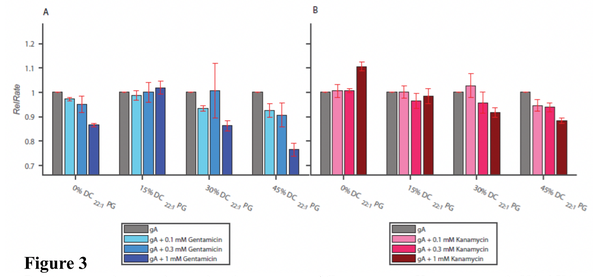


Figure 3

Relative quench rates observed in the presence of Gentamicin or Kanamycin with 0%, 15%, 30%, and 45% $DC_{22:1}PG$ lipids in the LUV membranes. Gentamicin (A) and Kanamycin (B) were tested at concentrations of 0 mM, 0.1 mM, 0.3 mM, and 1 mM.

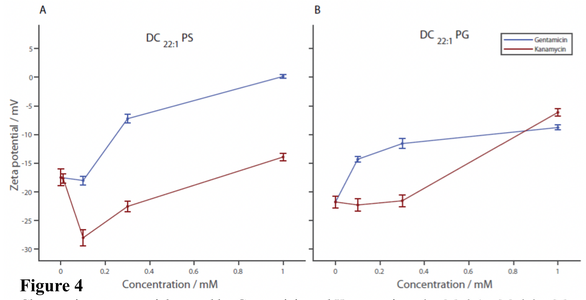


Figure 4

Changes in zeta potential caused by Gentamicin and Kanamycin at 0 mM, 0.1 mM, 0.3 mM, and 1 mM. (A) For $DC_{22:1}PS$ + $DC_{22:1}PC$, the zeta potential increased with Gentamicin concentration, but showed a biphasic (initial decrease followed by an increase) with Kanamycin. (B) For $DC_{22:1}PG$ + $DC_{22:1}PC$, the zeta potential for Gentamicin had the same trend, but the Kanamycin did not decrease significantly at 0.1 mM.

- Both Gentamicin and Kanamycin are polar molecules, as also evident by their calculated octanol/water partition coefficients. For Gentamicin and Kanamycin, AlogP are -3.81 and -7.14, respectively (calculated using Canvas in Schrödinger, LLC). This means neither is likely to partition into neutral vesicles (pure $DC_{22:1}PC$); however, because they are positively charged, they are more likely to partition into (or bind to) the head groups in the negatively charged ($DC_{22:1}PG$ and $DC_{22:1}PS$) vesicles.
- For the 30% $DC_{22:1}PS$ LUVs, the RelRate of Kanamycin decreased with increasing concentration, suggesting that the energetic cost to produce conducting channels with dimerization increases with increasing antibiotic concentration.
- Similarly, in 45% $DC_{22:1}PS$, the RelRate for 0.1 mM Kanamycin was undetectable, acknowledged by the asterisk where the bar should've been (**Fig. 2**). The lack of quenchable vesicles at this specific concentration may be related to the decrease in zeta potential at 0.1 mM Kanamycin in the $DC_{22:1}PS$ LUVs. I do not understand why this is the case, but I would like to explore it going forward.
- When I explored the antibiotics' effects on the zeta potential, the zeta potential in 45% $DC_{22:1}PS$ was roughly -18.0 mV and about -22.0 mV in 45% $DC_{22:1}PG$ (**Fig. 4**) in the absence of any aminoglycoside.
- As the aminoglycoside concentration increased, the zeta potential increased to become close to zero, most likely representing a decrease in negative surface charge of the liposomes due to their binding to the positively charged aminoglycosides (Au et al, 1986).

CONCLUSIONS

- I have shown that the aminoglycoside antibiotics alter the properties of lipid bilayers, as sensed by gramicidin channels.
- As the Gentamicin concentration increased, the charge on the negatively-charged bilayers was neutralized, which will decrease the likelihood that gramicidin would form channels.
- Gentamicin and Kanamycin have similar structures but differ in their maximal possible charge (+5 and +4, respectively); they both have subtle effects on gramicidin channel function (the Tl+ -induced quench rate) in negatively charged bilayers, which could result from changes in lipid bilayer elasticity or thickness.
- The aminoglycosides did not have any significant effects on the neutrally-charged vesicles; therefore, they didn't bind to the leaflet, as expected.
- Using DLS, I found that Gentamicin had a neutralizing effect on the charge of the lipid bilayer whereas Kanamycin increased the negative charge of the bilayer at 0.1 mM before beginning to neutralize at higher concentrations.

REFERENCES

1. Alberts, Bruce. "The Lipid Bilayer." *Molecular Biology of the Cell. 4th Edition.*, U.S. National
2. Library of Medicine, 1 Jan. 2002, www.ncbi.nlm.nih.gov/books/NBK26871/.
   Andersen, Olaf S., and Roger E. Koeppe. "Bilayer Thickness and Membrane Protein Function:
3. An Energetic Perspective." *Annual Review of Biophysics and Biomolecular Structure*, 2007, www.academia.edu/15483368/Bilayer_thickness_and_membrane_protein_function_an_en ergetic_perspective.
4. Andersen, Olaf S., et al. "Gramicidin Channels: Versatile Tools." *Biological Membrane Ion Channels: Dynamics, Structure, and Applications*, Springer, 2011, pp. 33–80.
5. Au, S, et al. "Membrane Perturbation by Aminoglycosides as a Simple Screen of Their Toxicity." Antimicrobial Agents and Chemotherapy, U.S. National Library of Medicine, Sept. 1986, www.ncbi.nlm.nih.gov/pmc/articles/PMC180567/.
6. Chung, L, et al. "Interaction of Gentamicin and Spermine with Bilayer Membranes Containing Negatively Charged Phospholipids." *Biochemistry*, U.S. National Library of Medicine, 15 Jan. 1985, www.ncbi.nlm.nih.gov/pubmed/3978084.
7. Drew, Richard H. "Aminoglycosides." Edited by David C. Hooper and Allyson Bloom, *UpToDate*, 19 July 2018, www.uptodate.com/contents/aminoglycosides.
8. Ganesan, Purushothaman, et al. "Ototoxicity: A Challenge in Diagnosis and Treatment." *Journal of Audiology & Otology*, The Korean Audiological Society and Korean Otological Society, Apr. 2018, www.ncbi.nlm.nih.gov/pmc/articles/PMC5894487/.
9. Gosline, John, et al. "Elastic Proteins: Biological Roles and Mechanical Properties." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, U.S. National Library of Medicine, 28 Feb. 2002, www.ncbi.nlm.nih.gov/pmc/articles/PMC1692928/.
10. Huth, M. E., et al. "Mechanisms of Aminoglycoside Ototoxicity and Targets of Hair Cell Protection." *International Journal of Otolaryngology*, Hindawi, 25 Oct. 2011, www.hindawi.com/journals/ijoto/2011/937861/.
11. Ingolfsson, Helgi I., and Olaf S. Andersen. 2010. 'Screening for Small Molecules' Bilayer- Modifying Potential Using a Gramicidin-Based Fluorescence Assay', Assay and Drug Development Technologies, 8: 427-36.
12. Ingolfsson, Helgi I., et al. "Phytochemicals Perturb Membranes and Promiscuously Alter Protein Function." ACS Chemical Biology, 5 June 2014, www.pubs.acs.org/doi/abs/10.1021/cb500086e/
13. Rusinova, Radda, et al. "A General Mechanism for Drug Promiscuity: Studies with Amiodarone and Other Antiarrhythmics." *The Journal of General Physiology*, The Rockefeller University Press, Dec. 2015, www.ncbi.nlm.nih.gov/pmc/articles/PMC4664825/.
14. Selimoglu, Erol. "Aminoglycoside-Induced Ototoxicity." Current Pharmaceutical Design, U.S. National Library of Medicine, 2007, www.ncbi.nlm.nih.gov/pubmed/17266591.