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

Biochemistry

RESEARCH PLAN

a. RATIONALE:

Aminoglycosides have been of a large importance since streptomycin was first clinically used in 1944 in World War II. The significance of aminoglycosides has since been reevaluated due researchers' want to reassess the basis of their functionalities to improve these drugs rather than simply inventing newer prototypes. This gramicidin-based fluorescence assay has been used to study many different bilayer properties but never used to investigate the toxicity of aminoglycosides. By conducting this novel research, I hope to provide researchers studying the reversibility and toxicity of these drugs with a unique perspective of their overall function and modifying capabilities. In the future, this research would benefit the pharmaceutical industry by allowing them to produce more effective and less toxic aminoglycosides by being able to predict which molecules are toxic before the millions of dollars of screening molecules. What nonspecific mechanisms are responsible to that are responsible for causing toxicity in bilayer? In the near future, I believe that there will be great advancements in my field of physics with applications in biology. Although we are currently limited in our understanding of the processes involving specific and nonspecific mechanisms, continuous research is being conducted to discover and understand the effects of changing membrane protein function. This newfound understanding could lead to benefits for those who suffer from ototoxic and nephrotoxic side effects such as permanent hearing loss and renal failure, which affects millions of people across the globe. Currently, researchers are reevaluating the changes of bilayer properties due to aminoglycoside antibiotic-induced toxicity to better understand these underlying mechanisms. Therefore, researchers could improve the efficacy of these aminoglycosides and limit the extent of the side effects. Ultimately, modifying these aminoglycosides will provide as a preventative measure to reduce the incidence of people with kidney damage and irreversible ear damage.

b. RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES:

My research introduced a novel mechanism to determine the extent of toxicity of a drug based on the fluorescence quench rates in the vesicles. Further research into the effects of altering bilayer properties will offer opportunities to investigate potential antibiotic treatment options. Similarly, as research continues to progress in the coming years, other aminoglycosides will be discovered, offering further opportunities for treatment.

c. PROCEDURES:

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) are from Avanti Polar

Lipids (Alabaster, AL). Methanol is 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) is from Sigma-Aldrich Co (St. Louis, MO). 8-Aminonaphthalene-1,3,6-Trisulfonic Acid, Disodium Salt (ANTS) is from Invitrogen (Eugene, OR). All materials will be used as received; all stock solutions of buffers and quenchers will be prepared before the experiment. Solutions will be dissolved in deionized water, at pH 7 with Sodium hydroxide (NaOH) and Nitric acid (HNO3) and stored at 12.5 °C. ANTS solution will be made with 25 mM ANTS, 100 mM NaNO3, 10 mM HEPES, and stored in the dark. Buffer will be made with 140 mM NaOH and 10 mM HEPES; quencher will be made with 50 mM TINO3 94 mM NaNO3 and 10 mM HEPES. 500 μg/mL of gD in powder form will be dissolved in methanol and stored at -40 °C.

Methods

*Large Unilamellar Vesicles (LUVs)*

The LUVs are going to be developed as described previously (Ingolfsson and Andersen, 2010; Rusinova et al., 2015). To make LUV stock solutions, I will equilibrate the lipids to room temperature after taking them from the freezer. I will prepare the control LUVs using 600 μL DC22:1PC. I will prepare LUVs consisting of DC22:1PC: DC22:1PS mixtures by combining 510 μL DC22:1PC and 90 μL DC22:1PS, 420 μL DC22:1PC and 180 μL DC22:1PS, or 330 μL DC22:1PC and 270 μL DC22:1PS to a test tube for 15%, 30%, and 45% DC22:1PC :DC22:1PS LUVs. For DC22:1PC: DC22:1PG mixtures I will combine 510 μL DC22:1PC and 90 μL DC22:1PG, 420 μL DC22:1PC and 180 μL DC22:1PG, and 330 μL DC22:1PC and 270 μL DC22:1PG to a test tube for 15%, 30%, and 45% DC22:1PG concentrations LUVs. With a total volume of 600 μL lipid in chloroform solution, I will add 30 μL gD (~1:2000) to the control and the 15% mixtures; 35 μL gD (~1:1800) to the 30% mixtures; and 40 μL gD (~1:1600) to the 45% mixtures. I will then dry the solution under nitrogen until all chloroform evaporates. The lipids will be further dried in a desiccator for at least 3 hours. The lipids will be rehydrated in 1.67 mL ANTS solution and covered in aluminum foil to shield from light. The sample will be equilibrated for 3 hours at room temperature and then sonicated for 1 minute, which will lead to the formation of multilamellar vesicles. The 6 freeze-thaw cycles (freezing in dry ice followed by thawing with water at ~55 °C); after the 6th cycle, the suspension will be extruded through an Avanti mini-extruder with a 0.1 μm polycarbonate filter. The suspension will be passed 43 times through the extruder’s filter. To remove external ANTS, the extruded suspension will be run over a PD-10 desalting column (GE Healthcare, Piscataway, NJ). The final stock LUV suspension contained 5 mM lipid and was stored in the dark at 12.5 °C. The LUV size distribution will be assessed using dynamic light scattering (DLS) for their diameters and polydispersity index (PDI).

*Stopped-flow Spectrofluorometry*

The rate of fluorescence quenching will be measured at 25 °C using a SX-20 stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, UK) in the single-mixing mode (**Fig. 4**). The excitation wavelength is set to 352 nm, the sampling rate to 5000 points/s, and the emission signal above 450 nm. Each experiment will be done as follows: first I will complete 8 mixing reactions for buffers and 10 repeats for quenchers. I will then dilute samples with buffer 100-fold to 50 μM LUVs. I will add the desired aminoglycoside-antibiotic concentration and incubate until the sample equilibrates at 25°C in the dark. Any deviant traces will be removed by visual inspection. Using SX-20 stopped-flow spectrofluorometry, I will measure the Tl+ influx rate through bilayer-spanning gramicidin channels. The ANTS-LUVs are mixed with amphiphiles, or other potentially bilayer-modifying drugs, and incubated at 25 °C until sample reaches equilibrium before mixing with either the buffer or the quench buffer. If the sample is mixed with the quencher the monovalent cations Tl+ will enter the vesicles and quench the encapsulated ANTS fluorescence.

*Dynamic Light Scattering (DLS)*

Vesicle size dispersity will be measured by DLS using a LitesizerTM 500 (Anton Paar, Austria). By using the Brownian motion model (with a wavelength of 658 nm, and a 90° detection angle, the testing temperature was set to 25 °C) and the Stokes-Einstein relation, I will calculate both the hydrodynamic diameter and PDI (Koppel 1972). 24 hours after extrusion, the amphiphile-free sample will be tested.

RISK AND SAFETY

Thallium is toxic and the onset of symptoms may occur over 12 to 24 hours. Thallium nitrate may be absorbed through the skin; readily absorbed through the digestive tract. May cause nausea, vomiting and diarrhea; weakness, numbness, and tingling of arms and legs. Loss of hair may occur after a few weeks. The risk is minimal with normal laboratory safety precautions. All scientists using thallium use gloves and lab coat; all thallium-containing solutions are aliquoted into Falcon tubes to minimize the risk of contamination. Falcon tubes, syringes and pipette tips used for handling thallium are disposed in appropriately labeled chemical waste containers. Gentamicin and Kanamycin are aminoglycosides and were handled only while using gloves and pipets as well as safety precautions.

DATA ANALYSIS

Each quench trace will be analyzed separately using MATLAB R2019a (The MathWorks Inc.) and quench rates were determined by fitting to a Stern-Volmer modified stretched exponential. Using the Smoluchowski model, I will also measure the mean zeta potential using DLS (Kirby and Hasselbrink 2004).

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