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Resurrecting the Dead: Mitigating Efflux-Pump Inhibitor Toxicity Using a Liposomal Delivery System to Recover Efficacy of Antimicrobial Drugs

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Abstract

Introduction: Antimicrobial resistance (AMR) has become concerningly prevalent on a global scale as many infectious agents have evolved to evade antimicrobials effects, making it difficult to treat infectious diseases. *Pseudomonas aeruginosa* is a multiresistant bacteria that requires urgent attention as it is detrimental in lung infections. Activity of membrane-embedded efflux pumps, such as the MexAB-OprM pump, is a principal mechanism by which bacterial species become resistant to antimicrobials. Efflux pump inhibitors (EPIs) have recently emerged as a strategy to prevent the expulsion of administered antimicrobials, thereby resensitizing resistant bacteria to antibiotics. Phenylalanine-arginine β-naphthylamide (PAβN) is an EPI that inhibits a number of different pumps, including the MexAB-OprM efflux system. Despite EPIs providing a partial solution to AMR, they have been shown to be toxic to humans, which has impeded their entry into clinical application. We propose that by inserting the PAβN into a liposomal delivery system, the cytotoxic effects against human cells will be lowered without decreasing the EPI's inhibitory activity.

Methods: Resistant *P. aeruginosa* strains will be administered with liposomes jointly encased with PAβN and ampicillin to ensure both are delivered to the same cell, while testing for changes in antimicrobial efflux activity and bacterial growth, indicative of restored antimicrobial effectiveness. Efflux activity and inhibited growth will be measured using an ethidium bromide efflux assay and a minimum inhibitory concentration assay, respectively. Human pulmonary epithelial cells will be exposed to liposomal-encased PAβN to study changes to EPI cytotoxicity, with cell viability being measured using a Cell Titer Blue assay.

Results: Liposomal EPI are expected to maintain inhibitory activity and resistant bacteria would become re-susceptible to antibiotics when treated with the liposomal EPI.

Discussion: By analyzing efflux rate to measure the liposomal EPI's activity, its activity level should be comparable to free EPI. The resensitization assay would show that the bacteria are susceptible to antibiotics again**.**

Conclusion: If effective, EPIs may become a potential therapeutic to combat AMR by reviving the use of antimicrobials that have become ineffective. Restoring the activity of already approved antibiotics through potential co-administration with liposome-encapsulated EPIs will be a cost-effective approach to combat AMR.

Keywords: antimicrobial resistance; efflux-pump inhibitors; liposomes; drug delivery; cytotoxicity; *Pseudomonas aeruginosa*; minimum inhibitory concentration

Introduction

Infectious agents, namely bacteria, viruses, and fungi are capable of invading cells and causing damage to various organisms, including humans [1]. This damage can be reduced through antimicrobials, however misuse and overuse has led to the emergence of antimicrobial resistance (AMR) [1]. According to the World Health Organization, one of the top 3 critical priority pathogens that requires the development of new antibiotics is *Pseudomonas aeruginosa*, which is known to cause fatal lung infections in patients with cystic fibrosis [2-4].

One mechanism for AMR in *P. aeruginosa* is through the overexpression of multidrug efflux pumps, which remove cytotoxic substances from infectious cells, making it difficult for antimicrobials to exert their effects [5,6]. *P. aeruginosa* intrinsically expresses the MexAB-OprM pump, an efflux pump system part of the Resistance-nodulation-cell division superfamily [5,6]. Consequently,

MexAB-OprM plays an important role in the emergence of AMR in *P. aeruginosa.*

Given that overexpression of efflux pumps is a prevalent mechanism by which bacteria develop AMR, efflux pump inhibitors (EPIs) have recently emerged as compounds that function as a competitive inhibitor of these pumps [7,8]. EPIs are designed to be co-administered alongside antibiotics, thereby preventing or decreasing the amount of antimicrobial drugs pumped out of the bacterial cell, which increases the efficacy of these drugs [9,10].

Phenylalanine-arginine β-naphthylamide (PAβN) is an EPI that inhibits a number of different bacterial efflux pumps, including the MexAB-OprM efflux system [11]. Despite the benefits of EPIs, they have been shown to be toxic to host cells, as evident through a number of *in vivo* studies, however the explanation for why EPIs can be toxic to human cells is still unknown [12]. This toxicity is the key barrier preventing the progression of EPIs to a clinical setting [13,14].

Improving drug delivery systems has recently surfaced as a unique approach to restore the activity of promising therapeutic compounds that present complications *in vivo*. Specifically, liposomal drug delivery is a promising strategy, as many liposome encapsulated drugs are currently undergoing clinical trials [15-17]. Liposomes act as an effective vehicle to bring active compounds to their target site, therefore increasing drug bioavailability [18,19]. This allows liposomes to decrease the dosage required to achieve a therapeutic effect and therefore improve the safety profile of these drugs.

Research Hypothesis

If PAβN is encapsulated within dipalmitoyl-phosphatidylcholine (DPPC)-cholesterol liposomes and co-administered with ampicillin, then the antibiotic will be able to inhibit the growth of resistant *P. aeruginosa* strains without cytotoxic effects from the EPI to human pulmonary epithelial cell viability.

Rationale

Since EPIs are promising therapeutic compounds that may help to overcome bacterial resistance to antibiotics, it is worthwhile to explore ways to improve their delivery. Using a liposomal delivery system is a promising technique to reduce drug toxicity, given its recent success in improving the bioavailability of other drugs and decreasing the levels required to achieve a pharmacological effect.

Methods

Liposome Encapsulation

Since neutrally-charged liposomes are known to have higher bioavailability in plasma [20], neutral liposomes will be created by mixing dipalmitoylphosphatidylcholine and cholesterol in a 6:1 ratio using the dehydration-rehydration method as follows: the DPPC-cholesterol mixture will be dissolved in a solution of chloroform and methanol at a 2:1 ratio; after evaporating the organic solvent using a rotary evaporator (Rotavapor), a liposome layer will form. PAβN will be inserted into the liposome through sonication (Figure 1) [21]. Using dynamic light scattering, the sizes of the liposomes can be analyzed, which can also verify if they were successfully generated. Liquid chromatography–mass spectrometry may then be used to verify that the drugs are encapsulated in the liposomes.

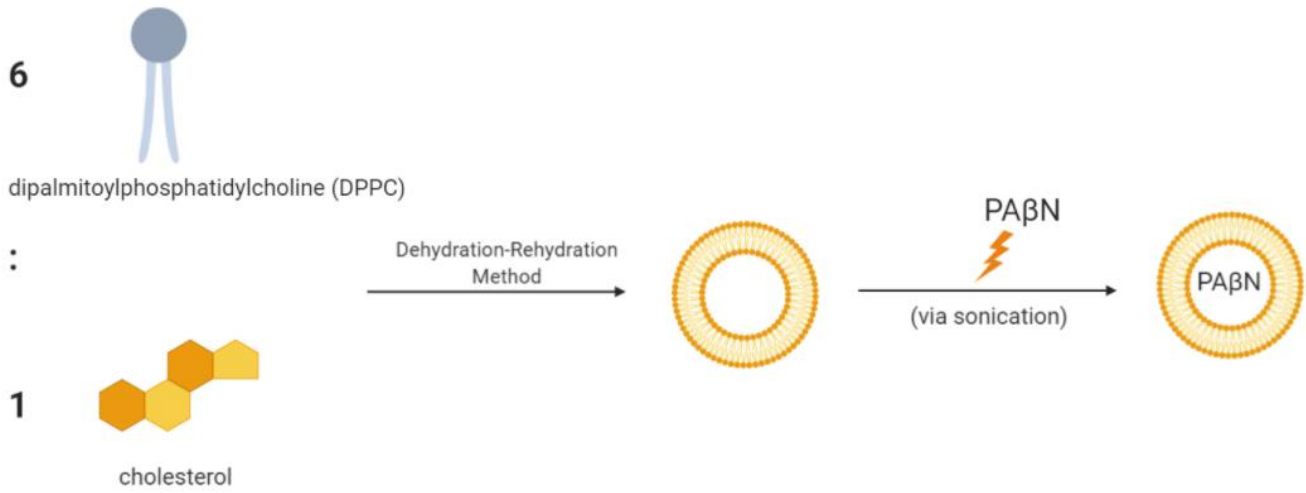

Figure 1. Liposome formation using a DPPC-cholesterol mixture, followed by the insertion of PAβN within the liposome. Figure created with BioRender.com.

Minimum Inhibitory Concentration (MIC) Assay

P. aeruginosa strain PA-Y7 is a highly resistant strain through the expression of MexAB-OprM [22]. Three cell cultures will be used: wildtype *P. aeruginosa*, MexAB-OprM overexpressing, and MexAB-OprM knockout (KO) cells. Cultures will be incubated in a 96-well plate with increasing concentrations (0, 125, 250, 500, and 1000 µg/mL) of ampicillin, produced by serial dilution with 0 µg/mL as the control. After a 24-hour incubation period at 37°C, each plate will be tested for inhibited growth by assessing optical density (OD) at 600 nm (OD600). On the plate, the antibiotic treatment will be done twice to account for within-plate variability, while the assay will be conducted as triplicates. Lower concentrations of ampicillin will still allow growth of *P. aeruginosa*, demonstrating the efflux abilities [23]. MexAB-OprM overexpressing strains will be expected to have a higher MIC than the wildtype strain (Figure 2), whereas knockouts will be expected to be more susceptible to ampicillin.

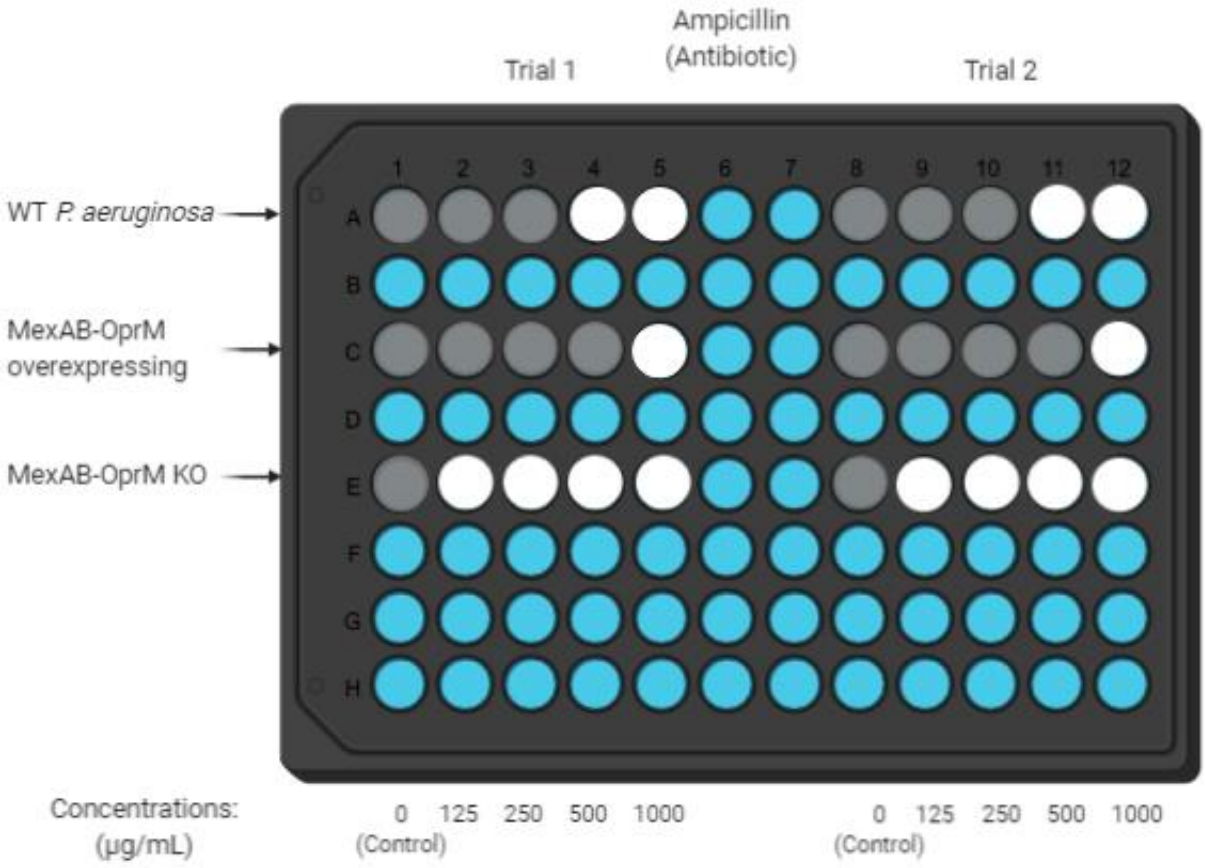

Figure 2. Expected MIC assay to assess the role of MexAB-OprM for the evolution of resistance in P. aeruginosa. Grey = bacterial growth; white = inhibited growth; blue = empty well. Figure created with BioRender.com.

Determining Liposomal EPI Cytotoxicity

Using a human pulmonary epithelial cell line (A549), a Cell Titer Blue assay will be used to determine cell viability [21]. Cells will be seeded into a 24 well plate in cell culture media with free or liposomal EPI at different concentrations, and cell culture media alone as the control. Cells are then left to incubate for 24 hours, before replacing the existing media with fresh media containing resazurin, a fluorogenic dye. Cell culture media with resazurin will be used as a baseline measure. Living cells can convert resazurin to fluorescent resorufin. Following this, fluorescence is measured to determine cell viability (Figure 3).

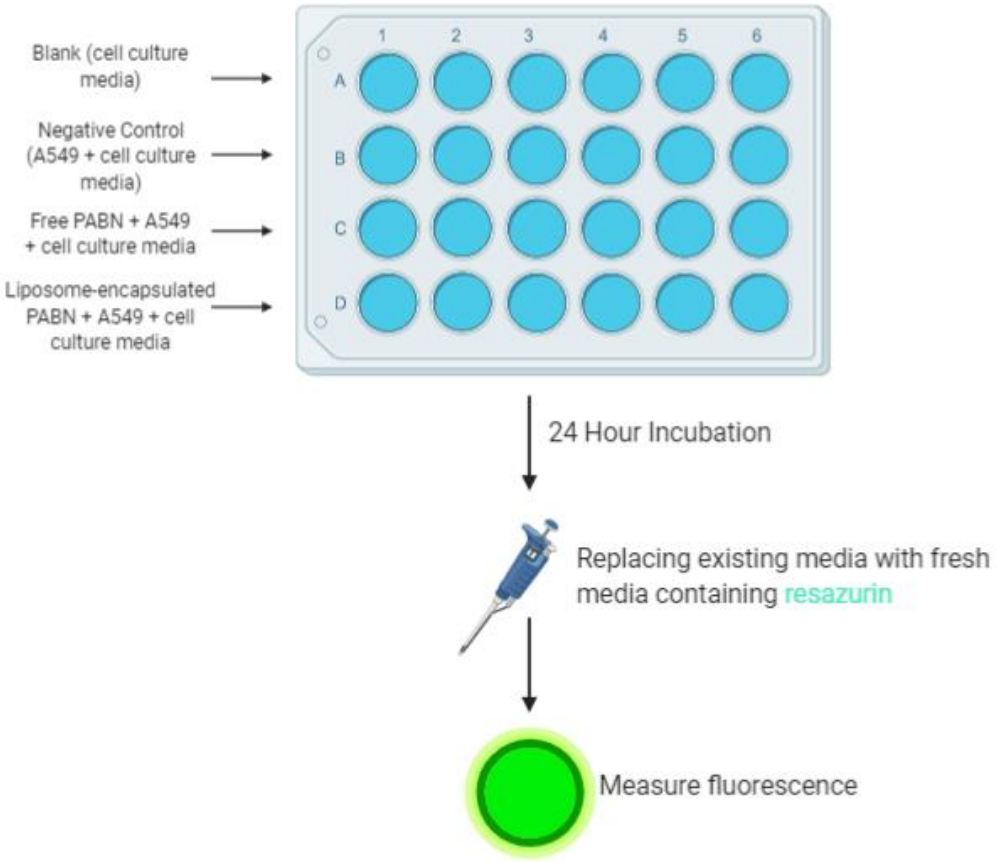

Figure 3. Cell Titer Blue assay to evaluate the liposomal PAβN's cytotoxic effect on human cells. A549 is a human pulmonary epithelial cell line and cell viability is determined using fluorescence, which will be measured using a fluorimeter [24]. Figure created with BioRender.com.

Measuring Inhibition of Pump's Efflux Activity

Using *P. aeruginosa* expressing MexAB-OprM grown in media, a protonophore, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), is added to temporarily stop the pumps [24,25]. Following this, one of three treatment groups will be administered: control (only ethidium bromide (EtBr)), free EPI with EtBr or liposomal EPI with EtBr. The control group allows determination of baseline activity of MexAB-OprM by fluorescence since MexAB-OprM can use this fluorescent dye as a substrate [26]. Cells are then resuspended in fresh media without CCCP to reverse the temporary inhibition [24,27] and the change in fluorescence is measured as the pumps are expelling EtBr out of the cells. Inhibition is shown if the rate of efflux is lowered.

Resensitizing Cells to Ampicillin using Liposomal EPI

As the MIC assays would show that the *P. aeruginosa* cells expressing MexAB-OprM were resistant to antibiotics, cells would be plated with ampicillin at a concentration below the MIC [28]. Liposomal PAβN at a concentration determined by the EtBr efflux assay to inhibit the pumps would also be added. There will be two controls: a blank control with media alone and a negative control with cells in media. Cells would be incubated at 37°C for 24 hours before OD is measured at 600nm. Results would show if the liposomal PAβN made the bacteria susceptible to the coadminstered antibiotics again.

Results

The expected results are that MexAB-OprM overexpressing *P. aeruginosa* strains will be expected to have a higher MIC than the wildtype strain (Figure 3) whereas knockouts will be expected to be more susceptible to ampicillin, and encapsulating the EPIs in liposomes would reduce cytotoxicity to the human pulmonary epithelial cell line. In addition, liposomal EPI would maintain inhibitory activity and resistant bacteria would become susceptible to antibiotics once again when treated with the liposomal EPI.

Discussion

Interpreting the expected results of the MIC assay, higher optical density would represent increased bacterial growth and resistance to ampicillin, whereas lower optical density would represent inhibited growth and susceptibility to ampicillin. When determining the liposomal EPI's cytotoxicity, fluorescence will indicate if the delivery system reduces cytotoxicity to human cells, as fluorescence will be observed within the results. Next, to measure the liposomal EPI's activity, the rate of efflux will be analyzed to observe if it is lowered compared to a control and has similar activity to free EPI. Lastly, the results of the resensitization assay would be interpreted to show that the bacteria are susceptible to antibiotics again. This would be done if the bacteria are shown to die when in combination with the liposomal EPI and the sublethal dose of antibiotics, based on the OD600.

Since this study is strictly an *in vitro* study, many limitations involve *in vivo* considerations. For example, there may be challenges regarding pharmacokinetic parameters, such as absorption and distribution of the EPI-liposomal complex. Future experiments will work to study the interaction of this complex with all cellular components, including pharmacokinetic and pharmacodynamic parameters. Specifically, future experiments can be done in intestinal cell lines to further understand this approach of combating *P. aeruginosa* infections. Moreover, testing the combination of liposomal EPI and penicillin in porcine animal models, a useful model for cystic fibrosis, will better inform the clinical applications of this approach [29].

Conclusions

Overall, we expect to see MexAB-OprM pumps conferring improved resistance to *P. aeruginosa*, liposomal EPIs reducing cytotoxicity, maintenance of liposomal EPI inhibitory activity, and finally, seeing resistant bacteria become susceptible to antibiotics once again. If EPI encapsulation into liposomes reduces cytotoxicity to human pulmonary epithelial cells, further testing may be done using different antimicrobial, EPI, and cell-line combinations to determine which EPIs could have their effectiveness revived. In addition, animal models may be later used to test the safety and efficacy of this strategy. If successful, EPIs may become a potential therapeutic to combat AMR in infections by reviving the use of antimicrobials that have become ineffective. Restoring the activity of already approved antibiotics through potential co-administration with liposome-encapsulated EPIs may be a cost-effective and worthwhile approach to combat AMR.

List of Abbreviations Used

AMR: antimicrobial resistance
CCCP: carbonyl cyanide m-chlorophenylhydrazone
DPPC: dipalmitoylphosphatidylcholine
EPIs: efflux pump inhibitors
EtBr: ethidium bromide
KO: knockout
MIC: minimum inhibitory concentration
OD: optical density
OD600: optical density measured at 600nm
PAβN: phenylalanine-arginine β-naphthylamide
P. Aeruginosa: *Pseudomonas aeruginosa*

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study did not require ethics approval.

Authors' Contributions

PR: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.
KN: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.
TN: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.
SS: made contributions to the design of the study, drafted the manuscript, and gave final approval of the version to be published.

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