Inhibitory Effect of Zosteric Acid and Alginic Acid On Bacterial Adherence to Human Lung Epithelial Cell Lines

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On Bacterial Adherence to Human Lung Epithelial Cell Lines

Abstract:
Bacteria existing in biofilms present problems for effective antibiotic therapy due to the protective properties of exopolysaccharide matrices. *Pseudomonas aeruginosa* (*PA*) regularly colonizes lung epithelial cells in patients with Cystic Fibrosis [3]. Zosteric acid (*ZA*), a naturally occurring phenolic acid found in eelgrass [1], and related compounds such as symmetrical bisphenols composed of naphthoic and benzoic acids are known to inhibit bacterial adherence and biofilm formation [4]. *P. aeruginosa* naturally lives in biofilms in the body, so to simulate this environment in cell culture we introduced alginic acid (*AA*) to the system. It has been found in many experiments that alginic acid is the key component that produces the biofilm in which *P. aeruginosa* lives. The aim of this study is to evaluate the anti-adhesion properties of zosteric acid in the presence of alginic acid on *P. aeruginosa* adherence to human lung epithelial cells A549. Untreated (control), or pre-treated with either 1% weight/volume (wt/vol) alginic acid, 1% wt/vol zosteric acid, or both 1% wt/vol alginic acid and 1% wt/vol zosteric acid, *P. aeruginosa* (DiIC18 labeled; red; 1.0x10^6/mL) was added to lung cells (Calcein-AM labeled; green) grown on coverslips for 45 minutes. Non-adherent bacteria were removed by washing and five random fields of view were recorded (800x) using an inverted microscope mounted with a CCD camera. After thresholding to remove excess noise, the ratio of red to green fluorescent pixels was calculated and treated samples were compared to untreated controls to determine inhibition. Experiments were also done involving microplates. Adherent A549 cells were grown on microplates. On the microplate, untreated cells as well as A549 cells treated with 1% wt/vol alginic acid, 1% wt/vol zosteric acid, and both 1% wt/vol alginic acid and 1% wt/vol zosteric acid were measured. All of these cells were treated with *P. aeruginosa* for 1 hour, except for one
group of control A549 cells, media cells, and a measured set of untreated wells. The amount of adherent bacteria in each well was then measured. The results of both experiments show that zosteric acid inhibits *P. aeruginosa* when it is on its own. However, in combination with alginic acid, this inhibition is not seen. Future research will use new techniques to determine whether this holds true in other experimental situations.

**Introduction:**

Bacteria found in nature exist in biofilms rather than a planktonic state found in laboratory cultures. Biofilms consist of polysaccharides excreted by the bacteria, which can protect the bacteria from the environment. Biofilm formation poses many advantages to bacteria, including protection from antibiotics, evasion of host immune response, and oxygen, for bacteria requiring anaerobic conditions [3].

The stages of biofilm formation first start with adhesion. This is usually accomplished by receptors on the bacterial membrane. The bacteria express a mucoid phenotype and excrete polysaccharides to form a matrix, which allows for the adhesion of more bacteria to form a mature biofilm [5].

**Zosteric acid (Figure 1.):** is a nontoxic phenolic acid, which can be isolated from a marine plant called eelgrass [1, 2]. Experiments with zosteric acid have shown that it inhibited the adhesion of bacteria to surfaces of materials submerged in water and prevented bio-fouling. In these experiments, zosteric acid was either used to treat the water, or it was incorporated into the surface of the materials [2]. Zosteric acid has also been used to spray agricultural crops to prevent the adhesion of fungus spores and bacteria. It proved to be nontoxic to eukaryotic cells [4].
In order to more closely stimulate environmental conditions, alginic acid (Figure 2) was added to the system to produce a biofilm. Many experiments point to O-acetylation of alginate as being a key component of biofilms\textsuperscript{[4, 7]}. While there is some debate on the issue of whether alginate actually produces biofilms, scholars seem to be unanimous on the fact that alginate is needed to give the biofilm its unique structure and also in protecting the biofilm from antibiotics.

The aim of this experiment is to test if zosteric acid in the presence of alginic acid can prevent the adhesion of bacteria, in this case \textit{Pseudomonas aeruginosa}, to human cells. The cells used in this experiment are human lung epithelial cells called A549. The A549 cells were either left untreated, treated with just 1% wt/vol alginic acid, 1% wt/vol zosteric acid, or both 1% wt/vol alginic acid and 1% wt/vol zosteric acid. If zosteric acid in the presence of alginic acid can prevent the adhesion of \textit{P. aeruginosa}, the combination could potentially be used to prevent colonization, or to slow the progression of an existing infection, of \textit{P. aeruginosa} in patients with health conditions like Cystic Fibrosis, whose lungs are chronically colonized by these bacteria.
Method:

Preparation of A549

A549 cells were grown on coverslips in 10% Fetal Bovine Serum in RPMI 1640 (Cambrex, Walkersville, MD) media for three days or until the cells were confluent at 37˚C and 5% CO₂. The A549 cells were labeled green with Calcein-AM (Molecular Probes, Eugene, OR) and allowed to incubate with the probe for 20 minutes at 37˚C.

Preparation of P. aeruginosa

A nutrient broth was inoculated with P. aeruginosa culture and allowed to grow for 24 hours at 37˚C. The P. aeruginosa were then centrifuged for 8 minutes at 3500 rpm. The supernatant was removed and another wash was performed by filling the tube containing the pellet with Phosphate Buffered Saline (PBS—HyClone Logan, UT) and then centrifuging for 8 minutes at 3500 rpm. After this centrifugation, P. aeruginosa were labeled red with DiIC18 (Molecular Probes, Eugene, OR) and allowed to incubate with the probe for 20 minutes at 37˚C. Then labeled bacteria were centrifuged at 3500 rpm and resuspended in RMPI 1640 to remove excess probe. The bacteria were counted, and a dilution of 1.0 x 10^6 cells/ml was made. This was done by first pouring off the supernatant of the centrifuged tube and then making the pellet up to 1 ml. 90 µl of crystal violet (SigmaAldrich) were combined with 10 µl of P. aeruginosa. 10 µl of this solution was then loaded into the hemacytometer for counting.

Treatments

Four treatments were used in this experiment: control (untreated), alginic acid, zosteric acid, and alginic acid/zosteric acid. All treatments were made exclusively to the A549 cells. 1% wt/vol alginic acid and zosteric acid were used. These were made by adding .15 g of the powder form of these compounds to 15 mls of RPMI 1640.
Flow

The FCS2 system was thoroughly sterilized and then put together as shown in Figure 3. The cover slip was mounted onto the Bioptechs FCS2 perfusion chamber. The perfusion was then washed with RPMI 1640 for 5 minutes to remove excess probe. After washing, bacteria were added to the system and perfused at a flow rate of .4 ml/min for 45 minutes at 37°C. After 45 minutes, the coverslip was washed with RPMI 1640 for 5 minutes to remove non-adherent bacteria.

![Figure 3. Bioptechs FCS2 perfusion chamber.](image)

Five random fields were viewed with a Leica DMIRB inverted microscope mounted with a CCD camera and images were captured, one in the red channel and one in the green channel. ImagePro Plus was utilized to threshold the images to remove excess noise and to generate the ratio of red to green fluorescent pixels. ImagePro Plus was also used to merge the two images from the red and green channels to form one image with red bacteria and green A549 cells. Treated coverslips were compared to the untreated control to determine the adherence of *P. aeruginosa* to A549 cells.
Static Adherence Assays

Essentially the same experiment was done in flat-bottomed clear 96-well Corning polystyrene microplates. The top 4 rows of the microplate were grown with confluent A549 cells. *P. aeruginosa* was prepared as before, except that the dye used was fluorescein (SigmaAldrich, orange) instead of DiI. The first column of the microplate was left untreated. The second column had bacteria added to it, but otherwise was left untreated. The third column was treated with alginic acid, the fourth column was treated with zosteric acid, and the fifth column was treated with both alginic acid and zosteric acid. After all of the pretreatments, *P. aeruginosa* was added to columns 2-5 and sat for 1 hour. All wells were then washed 3 times with PBS and left with 100 µl of PBS in them. 3 wells were measured that contained only PBS and 3 more were measured which were empty. All wells had their absorbance measured with the microplate reader at 490 for 1 second.

**Results:**

Earlier, experiments were done to determine which concentration of zosteric acid was needed to inhibit bacterial adherence. It was found that 1% wt/vol zosteric acid provided the most inhibition.

The flow experiment found that with just zosteric acid bacterial adherence was inhibited. However, in combination with alginic acid the bacteria clumped; and adherence was not blocked.

With the absorbance experiment, consistent results were not seen. It was successfully calibrated but when adherent cells were put in combination with bacteria, all values were measured around the same as the control which was an empty well.

**Discussion and Conclusion:**

In deciding which treatments to use, a lot of emphasis was placed on having sufficient controls and running physiologically relevant tests. The four main treatments that were used
provided this. Using untreated A549 cells in combination with untreated *P. aeruginosa* gave a baseline for comparison of everything else. Running experiments with alginic acid and zosteric acid separately was important in order to analyze the effects of each compound. Testing the experiment with both together (A549 cells were first treated with alginic acid and then zosteric acid in order to provide physiological relevance) ultimately answered the questions under study.

Other treatments were run a few times before they were rejected. One was treating the A549 cells with alginic acid and zosteric acid simultaneously. This was seen as irrelevant because cystic fibrosis patients would have alginate present in the cell environment before the introduction of zosteric acid. The bacteria were also studied with the four treatments done on the lung cells and then added to untreated A549 cells. However, this was also rejected because, though interesting, the experiment was not physiologically relevant.

The results from flow indicate that zosteric acid inhibited bacterial adherence on its own, but in the presence of alginic acid clumping was seen and adherence was not inhibited. Interestingly, earlier experimenters who had worked with overproduced alginate noticed clumps in their samples\(^4, 7\). This suggests that the clumping seen in the flow experiments here may have been due to overexpression of alginate.

In order to avoid the clumping seen in the flow experiments, static adherence assays were introduced. However, reliable results were never produced for this method. Using DiI as the dye, fluorescence was tried using clear plates. This produced results with higher readings for empty wells than those with bacteria in them. At first it was thought that the wrong filter was being used, but eventually it was decided that the problem came from light bouncing off of the clear plates. Next we tried absorbance. The microplate reader was successfully calibrated using bacteria in suspension, fluorescein as the dye, and measuring absorbance at 490. However, once
an assay was attempted with adherent cells and bacteria, all of the wells gave the same reading due to the microplate reader not being able to detect differences in absorbance when all of the cells/bacteria are adherent.

In the future, this experiment could be continued using a slightly different method involving the microplates. A549 cells would still need to be grown to confluence on clear microplates. The experiment would be carried out the same way except for, at the end, a lysing agent such as a detergent would be used to transfer the adherent labeled bacteria to black plates. This should work better because the black plates should reduce much of the “white noise” that was being produced with the clear plates. Also, lung cells and bacteria would have to be transferred from clear plates to black because the growth of the cells could not be monitored if they were initially grown on black plates. Doing this further microplate experiment should answer the question of whether the clumping seen when alginic acid is added happens just under perfusion conditions or if it happens under all conditions.

This research is so important because, according to Hentzer, et al, “Cystic fibrosis (CF) is the most common inherited lethal genetic disorder in Caucasian populations[4].” One of the things that makes it so deadly is alginate[4, 7]. Unfortunately, most cystic fibrosis patients are victims to P. aeruginosa infection, a point at which their prognosis steeply declines. It is important that research in this area continues so that hopefully people with cystic fibrosis will be able to live longer lives.

References:


