**In Vitro Aminoglycoside Potentiation on Escherichia coli Persisters by Metabolic Stimulation**


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**Available Online: 25th October, 2016**

**ABSTRACT**

The impossibility of eradicating the reason of recurrence of common bacterial diseases is our impetus in this study. Recent studies proved that this recurrence is caused by a subpopulation of bacteria, called the persisters, which is not susceptible with any antibiotic treatment because of its quasi-dormant state. Bacterial cellular processes are completely shut down but it thrives with cellular respiration and translation – making it possible for aminoglycoside antibiotics to target the ribosome. However, aminoglycosides have weak bactericidal activity and is energy dependent. The addition of sugar acts as the intermediate metabolite and produce energy to increase aminoglycoside uptake through proton motive force. As aminoglycoside uptake is increased, the eradication of the bacterial cell also increased. Different combinations of aminoglycoside and sugar metabolites were added to assayed bacterial persisters and spotted in a Luria-Bertani Agar to determine the number of colonies and its colony forming unit. The *Escherichia coli* persisters have the highest number of colonies which increases proportionally with time, while *E. coli* persisters with metabolite – aminoglycoside combination has a decreasing number of colonies as time increases. Galactose – gentamicin combination has the least number of colonies formed, with 7, 2, 0 and 0 for the first, second, third and fourth hour, respectively. The combination of galactose with gentamicin resulted to a decrease of active bacterial cells and decrease of persister viability as contact time increases. Therefore, Galactose – gentamicin combination is the best option for effective eradication of *E. coli* persisters via metabolic stimulation.

**Keywords:** *Escherichia coli* persisters; aminoglycoside metabolic stimulation

**INTRODUCTION**

In any given colony of, say, one million bacterial cells, there are bound to be one or two individuals that will survive an onslaught of antibiotics. The phenomenon, first identified in the 1940s, is called bacterial persistence or multidrug tolerance. Unlike antibiotic resistance, which results from a genetic alteration that renders a bacterium invulnerable to particular drugs, persistence derives from a sort of cellular sleep. Normal cellular functions shut down, giving antibiotics—which typically target active processes, such as translation or transcription—nothing to attack. Sixty-five percent of hospital-treated infections are caused by recurrent infections. A recurrent infection is a symptomatic reactivation of a latent infection caused by bacterial persisters. Bacterial persistence is a state in which a sub-population of dormant cells (persisters) tolerates antibiotic treatment. The persisters, though dormant, are primed for metabolic uptake, central metabolism, and respiration. Persister metabolism is of particular importance as it influences entry into, maintenance of, and exit from this antibiotic tolerant state. Antibiotics are used as treatment for bacterial infections such as caused by *Escherichia coli*, but according to James Collins, only 99% of the causative bacteria or less is eradicated by antibiotic treatment. The other 1% is not susceptible to the treatment due to its quasi-dormant state. The aim of the study is to establish a complete strategic eradication of *E. Coli* bacterial persisters by stimulating these into an active state using a potentiated aminoglycoside combined with a sugar-metabolite. Currently, there are no viable means for eradicating persisters. A study by Allison showed that metabolic stimulation might enhance aminoglycosides against bacterial persisters by generation of proton – motive force (PMF) which facilitates aminoglycoside uptake in dormant bacterial cells specifically targeting its ribosomes. This study elaborates and substantiates the proven aminoglycoside specificity of eradicating these persisters via metabolic stimulation by determining the log colony forming unit of the bacterial persisters by manipulating the metabolite-antibiotic combinations. This would be one of the first few bold steps that would lead to producing new pharmaceutical products of enhanced antibiotic medications especially for the chronic and recurrent infections instead of spending research funds and years of study in finding a brand new medication. This would not only benefit pharmaceutical companies and research industries but also the people in general as there is an ideal eradication of these diseases which is wise, safe
and cost-effective. This study was specifically conducted to determine the number of colonies of *Escherichia coli* persisters; the number of colonies of *Escherichia coli* persisters after treatment to a metabolite (mannitol and galactose) plus aminoglycoside (streptomycin, amikacin and gentamicin) per hour for four hours; the colony forming unit (log CFU) of *Escherichia coli* after metabolite-aminoglycoside treatment for four hours.

**MATERIALS AND METHODS**

**Preparation**

**Procurement of Escherichia coli**

*Escherichia coli* was purchased from the University of San Carlos, Department of Biology, Cebu City, Philippines.

**Preparation of the Escherichia coli Persisters**

*Escherichia coli* was diluted in 1:1000 in 20ml Luria-Bertani (LB) broth in a 125 ml flask and was cultured at 37°C overnight. The overnight culture was diluted 1:1000 in 1 ml LB in culture tubes (17x100 mm) and was cultured on a multishaker (250 rpm) at 37°C for 2.5 h. Ampicillin was added to a final concentration of 100 microgram ml⁻¹, and was incubated with aeration at 37°C for 3 h, causing lysis of a subset of the population. Surviving cells were pelleted (10000 x g, 5 min, 10°C), and a white opaque liquid with thick consistency settled, and was resuspended in 25 ml LB. The cells were cultured with aeration at 37°C for 16-24 h and were tested for resistance to killing by ampicillin. The procedure was repeated four times.

**Preparation of the Luria-Bertani Broth**

LB broth was prepared by measuring in a single-pan analytical balance exactly 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Tryptone, yeast and NaCl were dissolved in a 950 ml deionized water. The pH of the medium was adjusted to 7.0 using 1N NaOH and volume was brought up to 1 liter. The broth was autoclaved for 20 minutes at 15 psi. Then, the solution was cooled, and the antibiotic (50µg / mL of Ampicillin) was added. Then, the LB broth was stored at 20 to 26°C.

**Preparation of the Luria-Bertani Agar**

LB broth was prepared by measuring in a single-pan analytical balance exactly 10 g tryptone, 5 g yeast extract, 15g agar and 10 g NaCl. Tryptone, yeast, agar and NaCl were dissolved in a 950ml deionized water. The pH of the medium was adjusted to 7.0 using 1N NaOH and volume was brought up to 1 liter. The broth was autoclaved for 20 minutes at 15 psi. Then, the solution was cooled, and the antibiotic (50µg / mL of Ampicillin) was added. Then the LB agar was stored at 20 to 26°C.

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**Collection and Preparation of Sugar Metabolites and Aminoglycosides**

The following sugar metabolites were used: 10 mM Mannitol and 10mM Galactose which were purchased from the Department of Pharmacy of the University of San Carlos. The aminoglycosides used were the following: 50µg/ml Streptomycin, 50µg/ml Amikacin, and 10µg/ml Gentamicin. These were purchased from a licensed drug store.

**Eradicating Bacterial Persisters by Metabolite-Aminoglycoside Combination**

One mL of *Escherichia coli* persisters was placed separately into four sterilized culture tubes. Then the first tube was labelled with 1 hour, followed by 2 hours on the next tube, 3 hours and 4 hours for the last two tubes, respectively. Each culture tube was added with 1 mL of metabolite and 1 mL of aminoglycoside with its specific concentration and placed on a multi shaker (250 rpm) at 37°C. First culture tube was removed after one hour and 10 µL was spotted on an agar plate for determination of colony-forming units. Second culture tube was removed after two hours and 10 µL was spotted on an agar plate for determination of colony-forming units. Third culture tube was removed after 3 hours and 10 µL was spotted on an agar plate for determination of colony-forming units. Fourth culture tube was removed after 4 hours and 10 µL was spotted on an agar plate for determination of colony-forming units using a colony counter.

**Spotting of Test Solutions on LB Agar**

A micropipette was used to drop 10 microliters of each of the test solutions with *Escherichia coli* persisters from the culture tube to the LB agar. The glass spreader was sterilized and cooled. Using the sterilized glass spreader, the spotted solution was spread evenly on the surface of the plate. The plates were not disturbed for 10-20 minutes. After drying the plates, these were incubated for bacterial culture overnight.

**Spotting of Escherichia coli Persisters on LB Agar**

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**Determination of the Number of Colonies**

Each of the Luria-Bertani Agar, spotted with 10 microliters of test solution and *Escherichia coli* persisters, was placed on the colony counter. The number of colonies was manually counted.

**Calculation of Colony Forming Unit per one mL of *E. coli* Persisters**

\[
\text{Colony-Forming Unit/mL} = \frac{\text{CFU/ml}}{\text{Volume of culture plate}}
\]

\[
\text{Log (CFU/ml)} = \text{Log Value} = \log \left( \frac{\text{CFU}}{\text{ml}} \right)
\]

**RESULTS**

*Escherichia Coli Persisters*
The number of colonies of *E. coli* persisters are 33, 38, 44, and 47 for the first, second, third and fourth hour, respectively. The *E. coli* persisters continue to increase as time increases. The number of colonies of *E. coli* persisters with aminoglycosides increases as time increases (Fig. 1).

**Metabolite-Streptomycin Combination**

**Mannitol-Streptomycin Combination**

The average numbers of colonies formed for Mannitol-Streptomycin combination: 15 (1 hour), 12 (2 hours), 3 (3 hours), 1 (4 hours). The average CFU for the Mannitol-Streptomycin combination: 1500 (1 hour), 1200 (2 hours), 300 (3 hours), 100 (4 hours). The average log CFU for the Mannitol-Streptomycin combination: 3.17 (1 hour), 3.07 (2 hours), 2.48 (3 hours), 2 (4 hours).

**Galactose-Streptomycin Combination**

The average number of colonies formed for Galactose-Streptomycin combination: 7 (1 hour), 5 (2 hours), 2 (3 hours), 0 (4 hours). The average CFU for the Galactose-Streptomycin combination: 700 (1 hour), 500 (2 hours), 200 (3 hours), 0 (4 hours). The average log CFU for the Galactose-Streptomycin combination: 2.85 (1 hour), 2.70 (2 hours), 2.30 (3 hours), 0 (4 hours).

**Streptomycin without Metabolite**

The average number of colonies formed for Streptomycin without Metabolite: 17 (1 hour), 21 (2 hours), 24 (3 hours), 27 (4 hours). The average CFU for the Streptomycin without Metabolite: 1700 (1 hour), 2100 (2 hours), 2400 (3 hours), 2700 (4 hours). The average log CFU for the Streptomycin without Metabolite: 3.23 (1 hour), 3.32 (2 hours), 3.38 (3 hours), 3.43 (4 hours).

**Metabolite-Gentamicin Combination**

**Mannitol-Gentamicin Combination**

The average number of colonies formed for Mannitol-Gentamicin combination: 10 (1 hour), 5 (2 hours), 3 (3 hours), 0 (4 hours). The average CFU for the Mannitol-Gentamicin combination: 1000 (1 hour), 500 (2 hours), 300 (3 hours), 0 (4 hours). The average log CFU for the Mannitol-Gentamicin combination: 3 (1 hour), 2.70 (2 hours), 2.48 (3 hours), 0 (4 hours).

**Galactose-Gentamicin Combination**

The average number of colonies formed for Galactose-Gentamicin combination: 5 (1 hour), 2 (2 hours), 0 (3 hours), 0 (4 hours). The average CFU for the Galactose-Gentamicin combination: 500 (1 hour), 200 (2 hours), 0 (3 hours), 0 (4 hours). The average log CFU for the Galactose-Gentamicin combination: 2.70 (1 hour), 2.30 (2 hours), 0 (3 hours), 0 (4 hours).

**Gentamicin without Metabolite**

The average number of colonies formed for Gentamicin without Metabolite: 16 (1 hour), 21 (2 hours), 26 (3 hours), 33 (4 hours). The average CFU for the Gentamicin without Metabolite: 1600 (1 hour), 2100 (2 hours), 2600 (3 hours), 3300 (4 hours). The average log CFU for the Gentamicin without Metabolite: 3.2 (1 hour), 3.32 (2 hours), 3.41 (3 hours), 3.52 (4 hours).

**Metabolite-Amikacin Combination**

**Mannitol-Amikacin Combination**

The average number of colonies formed for Mannitol-Amikacin combination: 11 (1 hour), 9 (2 hours), 3 (3 hours), 0 (4 hours). The average CFU for the Mannitol-Amikacin combination: 1100 (1 hour), 900 (2 hours), 300 (3 hours), 0 (4 hours). The average log CFU for the Mannitol-Amikacin combination: 3.04 (1 hour), 2.95 (2 hours), 2.48 (3 hours), 0 (4 hours).
**Galactose-Amikacin Combination**

The average number of colonies formed for Galactose-Amikacin combination: 8 (1 hour), 6 (2 hours), 1 (3 hours), 0 (4 hours). The average CFU for the Galactose-Amikacin combination: 800 (1 hour), 600 (2 hours), 100 (3 hours), 0 (4 hours). The average log CFU for the Galactose-Amikacin combination: 2.90 (1 hour), 2.78 (2 hours), 2 (3 hours), 0 (4 hours).

**Amikacin without Metabolite**

The average number of colonies formed for Amikacin without Metabolite: 17 (1 hour), 21 (2 hours), 26 (3 hours), 33 (4 hours). The average CFU for the Amikacin without Metabolite: 1700 (1 hour), 2100 (2 hours), 2600 (3 hours), 3300 (4 hours). The average log CFU for the Amikacin without Metabolite: 3.23 (1 hour), 3.32 (2 hours), 3.41 (3 hours), 3.52 (4 hours).

**Metabolite-Aminoglycoside Combination**

Aminoglycosides with metabolites displayed an enhancement in eradicating *E. coli*. Aminoglycosides without metabolites, did not display an enhancement on eradicating *E. coli* persisters, instead, showed a continuous increase in the number of colonies because of its high colony forming unit or its viability. Thus, metabolites have a significant role in increasing the uptake of Aminoglycoside drugs resulting to an enhanced elimination of *E. coli* persister cells. Among all the combinations, only Galactose – Gentamicin combination displayed a fast rate of a declining number of colonies, the least number of colonies and colony forming unit, and a complete eradication of bacterial persister cells. Therefore, Galactose – Gentamicin is the best combination that can completely enhance aminoglycoside uptake to completely eradicate *Escherichia coli* persisters.

**DISCUSSION**

A small subset of the bacterial population is not susceptible to medical treatment because of its quasi-dormant state. The eradication of bacterial persisters is possible only by metabolic stimulation since these are capable of translation even if most cellular processes are completely shut down. Aminoglycosides are the best option for this experiment as it targets the cell’s ribosome. However, aminoglycosides have weak activity against this subpopulation of cells and its uptake into a bacterium is energy dependent. Sugar metabolites enhance the eradication of the surviving bacterial cells by stimulating its metabolism.

The *E. coli* persisters were treated, in both the presence and absence of sugar metabolites, with a member of the aminoglycoside antibiotics: Gentamicin, Streptomycin, and Amikacin. Sugar metabolites were screened for their ability to potentiate against *E. coli* persisters. These sugar metabolites (Galactose and Mannitol) were selected by their glycolysis pathway which they enter the upper glycolysis through the pentose-phosphate pathway (PPP) and entner-duoderoff pathway (EDP). Persisters were resuspended to an LB broth supplemented with specific metabolite-aminoglycoside combination. Metabolite-aminoglycoside combination showed eradication of bacterial persisters. Among the three aminoglycosides, the Galactose-Gentamicin combination was greatly potentiated by a sugar metabolite against persisters than the two other combination (Metabolite-Amikacin and

![Figure 3: Metabolite – Gentamicin Combination](image3)

![Figure 4: Metabolite – Amikacin Combination](image4)
Metabolite-Streptomycin. The Galactose-Gentamicin combination showed the lowest CFU among the other metabolite-aminoglycoside combination. Metabolites entering the upper glycolysis (Galactose and Mannitol) induced rapid gentamicin eradicating persisters thus reducing persister viability. No eradication was observed on the media in the absence of added metabolite thus treated cells were persistent to aminoglycosides. Gentamicin like the other members of its group has a broad spectrum of activity against both gram-negative and gram-positive bacteria. Of particular interest is its strong activity against gram-negative enteric bacteria (E. coli). Amikacin needs higher dosages of amikacin for the treatment of most gram-negative bacillary infection. Streptomycin is known to be effective against the tubercle bacillus. Given the energy dependence of aminoglycoside uptake, the sugar metabolite screened increased aminoglycoside uptake. Metabolites that induced aminoglycoside killing were observed to induce high levels of aminoglycoside uptake, implying that increased uptake induced by these metabolites was responsible for aminoglycoside eradication. Aminoglycoside uptake requires a proton-motive force (PMF). These uptake facilitated by the metabolite promotes eradication of persister by aminoglycosides. These sugar metabolites which induced aminoglycoside uptake and eradication were the ones that elevate PMF. As the PMF increases the aminoglycoside uptake increases as well thus increases eradication of persister.9–12 These persister are primed for biochemical processes, such as central metabolism, that allow PMF induction. But these central metabolism and respiration in persisters are not enough to support other processes necessary for cellular growth such as cell-wall biogenesis and DNA replication. Thus, persisters treated with specific metabolites appear to be energized which facilitates their elimination by aminoglycosides. These metabolites are transported to the cytoplasm, some by their specific phosphotransferase system enzymes, and enter glycolysis, where their catabolism generates NADH. NADH is oxidized by enzymes in the electron transport chain, which in turn contribute to PMF. The elevated PMF facilitates the uptake of aminoglycosides which bind to the ribosome causing mistranslation-induced cell death.13–16 The Escherichia coli persisters have higher tolerance to diverse antibiotics. The number of E. coli colonies increase even after extended incubation times in the presence of antibiotics. Exposure to antibiotics for four times led to visible lysis of the culture thus increase persister levels. The number of Escherichia coli persisters decreases as the time of exposure to metabolite-aminoglycoside combination increases. The lesser is the CFU (Colony Forming Unit) value, the greater is the ability of a specific metabolite-aminoglycoside combination to eradicate E. coli persisters. The log CFU of E. coli persisters after treatment of metabolite-aminoglycoside combination based on the colony forming unit of each combination reflects the viability of the cell count in a colony. The lowest log CFU reading was from the Galactose-Gentamicin combination. The lower is the log CFU of a metabolite-aminoglycoside combination, the higher is the potentiation against E. coli persisters. Thus, Galactose – Gentamicin is the best combination to eradicate E. coli persisters.

CONFLICT OF INTEREST
The authors declare no conflict of interest

ACKNOWLEDGEMENTS
The researchers would like to express their gratitude to the University of San Carlos, School of Health Care Professions, Department of Pharmacy through the Chairman, Ms. Nelly Nonette M. Ouano and the Dean, Yolanda C. Deliman, for their unwavering support in the conduct of this study.

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