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Immuno-Anti-Infective Drug Design Using BioAI

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I HEREBY RECOMMEND THAT THE THESIS PREPARED

UNDER MY SUPERVISION BY

Cathy Chen and Vivian Zhang

ENTITLED

IMMUNO-ANTI-INFECTIVE DRUG DESIGN USING BIOAI

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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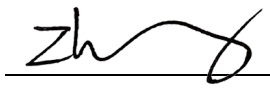

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IMMUNO-ANTI-INFECTIVE DRUG DESIGN USING BIOAI

By

Cathy Chen, Vivian Zhang

SENIOR DESIGN PROJECT REPORT

Submitted to
the Department of Bioengineering

of

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Abstract

According to the World Health Organization, antibiotic resistance is one of the biggest threats to global health, food security, and development today. A growing number of infections, like Methicillin-resistant Staphylococcus aureus, are becoming harder to treat as the antibiotics used to treat them become less effective. As a result, the primary concern for infections in the hospital setting is due to the *S. aureus's* growing resistance to antibiotics. Therefore, in response to this global health threat, our project focuses on furthering the research in developing a drug that *S. aureus* will not develop resistance to. In this paper, we assess NPY-Y2 as a potential immuno-anti-infective drug target to prevent the activation of Sortase A on *S. aureus.* We have shown that NPY-Y2 is a potential drug target; however, further invasion assay experiments need to be conducted for more reliable verification. In a larger scheme, our hope is that the approach of this research will allow for the development of other anti-infective drugs for other bacteria.

Acknowledgement

We would like to thank our advisor, Dr. Zhang, for his valuable advice and support throughout the project. We would also like to thank Lisa Jin and Melina Huang Xia for the training and support with the invasion assay. We would also like to acknowledge Daryn Baker, Victoria Walton, and Wendy Raposa for their support in the lab, as well as Anna Fisher and Maria Esquivel for their help with mammalian cell culture. Finally, thank you to the Santa Clara University School of Engineering for the financial support.

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List of Abbreviations

Artificial Intelligence	AI
Bioengineering	BIOE
Carboxyfluorescein succinimidyl ester	CFSE
Chinese Hamster Ovary	CHO
Deionized	DI
Dimethyl Sulfoxide	DMSO
Dulbecco's Modified Eagle Medium	DMEM
Fluorescence-Activated Cell Sorter	FACS
Fetal Bovine Serum	FBS
Food and Drug Administration	FDA
Methicillin-Resistant *Staphylococcus aureus*	MRSA
Monoclonal Antibody	mAb
Multiplicity of Infection	MOI
Tryptic Soy Broth	TSB

Chapter 1: Introduction

Background

Antibiotics prevent and treat bacterial infections; therefore, antibiotics are vitally important in healthcare for invasive procedures and in the food industry to treat livestock. However, the effectiveness of antibiotics is threatened by bacteria's ability to develop resistance to the drug. Antibiotic resistance is a crisis that needs to be addressed by developing a new approach to prevent and treat bacterial infections. Our research project strives to advance the development of a non-antibiotic therapy to alleviate the consequences of antibiotic resistance.

Literature Review

Antibiotics

Antibiotics were arguably the greatest achievement within the medical community of the 20th century. Since the discovery of antibiotics, the average human lifespan has increased 23 years (Hutchings, et. al., 2019). German scientist, Dr. Paul Ehrlich, played a prominent role behind the discovery of the first antibiotic drug in 1910, a synthetic derivative from dyes (Hutchings, et. al., 2019). Dr. Ehrlich reasoned that because the immune system was chemically driven, it should be possible for us to develop a chemical that could specifically interact with only a disease-causing organism. He called this chemical therapy (or chemotherapy drug) a "magic bullet" - a bullet that kills only the targeted organism (Hutchings, et. al., 2019). Another approach to antibiotic development was proposed by Louis Pasteur in the 19th century based on his observations of antibiosis (Kong, et. al., 2009). Antibiosis is the death or inhibition of growth of microbes due to toxic molecules released by other microbes for protection or predation. Pasteur proposed that these molecules can be identified and isolated to be used as drugs to combat infectious diseases. In 1928, Alexander Fleming discovered penicillin, the first natural antibiotic (Kong, et. al., 2009). The discovery of penicillin supported Pasteur's proposed theory of a natural antibiotic drug and catalyzed the systematic study of microbes to discover other classes of natural antibiotics to combat microbial infections.

Many of the natural antibiotic drugs discovered during this era are still being used today and have made many medical procedures possible. Antibiotics are used as both a treatment for infection and for prevention. Antibiotics are necessary as prevention for those with a weakened immune system, those taking immunosuppressive drugs, cancer patients, and for invasive procedures (Hutchings, et. al., 2019).

Current antibiotics work by targeting unique bacterial molecules that aren't found in mammalian cells but are crucial for bacteria survival. These target molecules are involved in cell wall synthesis, protein synthesis, DNA replication, DNA transcription, and metabolic processes (Hutchings, et. al., 2019).

Antibiotic Research and Development

There are two general types of antibiotics - natural product and synthetic (Hutchings, 2019). Natural antibiotics are found by screening for molecules that exist naturally in our body, and they are either part of the immune system or have the function of antibiotics. Synthetic antibiotics are made in the laboratory solely with known functional structure and design. Sometimes, synthetic antibiotics are discovered by modifying the structure of natural molecules. In the mid-twenty century, during the golden age of antibiotic discovery, researchers established the current categories of antibiotics (Hutchings, 2019). Some contemporary approaches include: "open innovation; targeting specific pathogens and/or specific organs in the patient; examining the effects of antimicrobial compounds on bacterial virulence as well as on antibiotic-resistant variants and searching for antibiotic producers among microorganisms not previously well explored" (Leisner, 2020). The drug discovery approaches researchers use today fall into one of the listed categories. There have been no major innovations on antibiotic mechanisms.

Antibiotic Resistance

The consequences of antibiotic resistance are multifaceted and will disproportionately impact countries and individuals differently. More than 2.8 million antibiotic-resistant infections and 35,000 deaths occur in the US each year (CDC, 2019). If this silent pandemic continues without intervention, it could result in 10 million deaths per year by 2050 (United Nation Foundation, 2021). The projected global economic burden caused by antibiotic resistance would increase

from $5 billion to $100 trillion (United Nation Foundation, 2021). The consequences of antibiotic resistance would be particularly damaging to low- and middle-income countries. The economies of these countries rely on agricultural productivity, which is also challenged by antibiotic resistance. Thus, antibiotic resistance risks widening the global health and economic inequality gap (United Nation Foundation, 2021).

Antibiotic resistance arises from the bacteria's tendency to mutate and from the process of natural selection. Under stress, like in the presence of antibiotics, colonies of bacteria can increase their rates of mutation (Peterson, et. al., 2018). This increases the likelihood of altering or removing the molecule that antibiotics are targeting. And prolonged colony exposure to antibiotics creates a pressure that naturally selects for bacteria with the mutation so that all the bacteria remaining in this colony are now resistant to antibiotics. In addition to altering or removing antibiotic target molecules, bacteria have also developed other antibiotic resistant genes that provide defense strategies against antibiotics. These defense strategies include restricting access of antibiotics by altering their membrane, removing antibiotics using pumps, and destroying the antibiotic using enzymes (CDC, 2021). Moreover, bacteria are able to share these antibiotic resistant genes to neighboring bacteria through horizontal gene transfer mechanisms: transformation, transduction, and conjugation (von Wintersdorff, 2016).

Knowing the defense mechanisms of bacteria against antibiotics, there are two major issues with current antibiotic mechanisms. One problem with current antibiotics is that it relies on targeting specific molecules of the bacteria. This is an issue because bacteria can mutate to change or remove these molecules. The second problem is that antibiotics either kills the bacteria or prevents the growth of the bacteria. This is a problem because then antibiotics act as the pressure for natural selection of antibiotic resistant bacteria. So, because of how antibiotics work, antibiotic resistance is inevitable.

Methicillin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is a bacterium that's naturally found on skin and in the nose of healthy individuals (CDC, 2020). It's classified as a gram-positive bacterium, so it has a thick protective coating of peptidoglycans making up its cell wall. *S. aureus* only becomes an issue when it enters

the body. Risks for staph infection include prolonged stays at the hospital, invasive procedures, unsanitary food preparations, contact sports, and underlying health problems that weaken the immune system (CDC, 2020). These risks are not completely avoidable, especially in places with poor sanitary conditions and high population densities. And with COVID-19, these risks are of great concern for hospitalized patients or intubated patients on the ventilators.

Methicillin-resistant *staphylococcus aureus* (MRSA) is a common and continual threat particularly in hospitals. Within the hospital setting, MRSA is the primary cause of infection and can lead to bloodstream infections, pneumonia, or surgical site infections. If left untreated, the infection can develop into sepsis, which has a mortality rate between 20% - 50% (CDC, 2019). Unfortunately, MRSA is incredibly difficult to treat because it has developed resistance to many first-line classes of antibiotics including methicillin, penicillin, and amoxicillin. In 2017, there were roughly 323,700 cases of MRSA and 10,600 deaths (CDC, 2019).

Facultative Intracellular Microorganism

Staph infection occurs following the adhesion and invasion of *S. aureus* into host cells. Therefore, *S. aureus* is classified as a facultative intracellular microorganism, a type of pathogen that can proliferate inside host cells. Depending on the tissue of the host cell, staph infection can lead to pneumonia in the lungs, septicemia in the blood, and meningitis in the brain, etc.

S. aureus is able to survive in the host environment and invade mammalian cells using a variety of virulence factors. These virulence factors include antibody presentation to camouflage the bacteria from immune cells, secreted toxins to suppress the immune response, cell surface adhesins that allow it to form biofilms and bind to tissues, and invasins to enter in and out of mammalian cells (Jenul, 2019). As a result, *S. aureus* can survive and proliferate inside both phagocytic immune cells (e.g. neutrophils) and non-phagocytic cells (e.g. endothelial cells, epithelial cells, osteoblasts) (Bongers, 2019). Anti-virulence strategies may be developed to prevent expression of disease-causing virulence factors. However, *S. aureus* utilizes a combination of a variety of virulence factors to attach and invade mammalian cells. Therefore, developing antibiotics to target one virulence factor is not a realistic strategy to treat staph

infection. So, to develop an anti-virulence therapy, the target needs to be an upstream regulator of virulence factor expression.

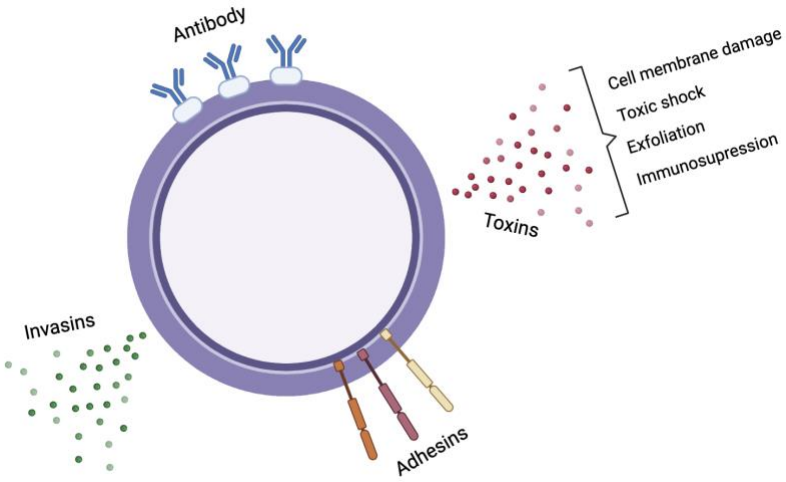

Figure 1. *S. aureus* virulence factors

Virulence Factor Expression Mechanism

Sortase A

Sortase is a transpeptidase enzyme found on the gram-positive cell wall of bacteria, including *S. aureus*. The most common sortase enzyme is Sortase A. The activation of Sortase A results in expression and covalent anchoring of invasion virulence factors onto the cell wall (Zhu, et. al., 2018). Proteins to be displayed on the cell wall are labeled with the LPXTG motif signal (Mazmanian, 1999). Sortase A cleaves the bond between threonine and glycine of the signaling motif (Mazmanian, 1999). Then it covalently links the threonine end of the protein to pentaglycine of the cell wall, displaying the protein onto the bacteria's cell surface. Sortase A is only found in bacteria; therefore, it can be a viable target for antibiotic targeting without risking damage to mammalian cell activity.

Regulation of Sortase A Activity

Research suggests that when Sortase A exists in a monomer confirmation, it expresses more virulence factors than its homodimer conformation (Zhu, 2015). Therefore, Sortase A activity is likely dependent on its conformation state in order to regulate energy expenditure on virulence factor display. So, when Sortase A is in a dimer conformation, it is inactive. Therefore, the

bacteria are unable to express virulence factors and can't invade the mammalian cell. Further research suggests that a binding mechanism induces Sortase A to undergo a conformational change from a dimer to a monomer conformation (Zhu, 2015). In this active monomer conformation, the bacteria can now express virulence factors and invade mammalian cells.

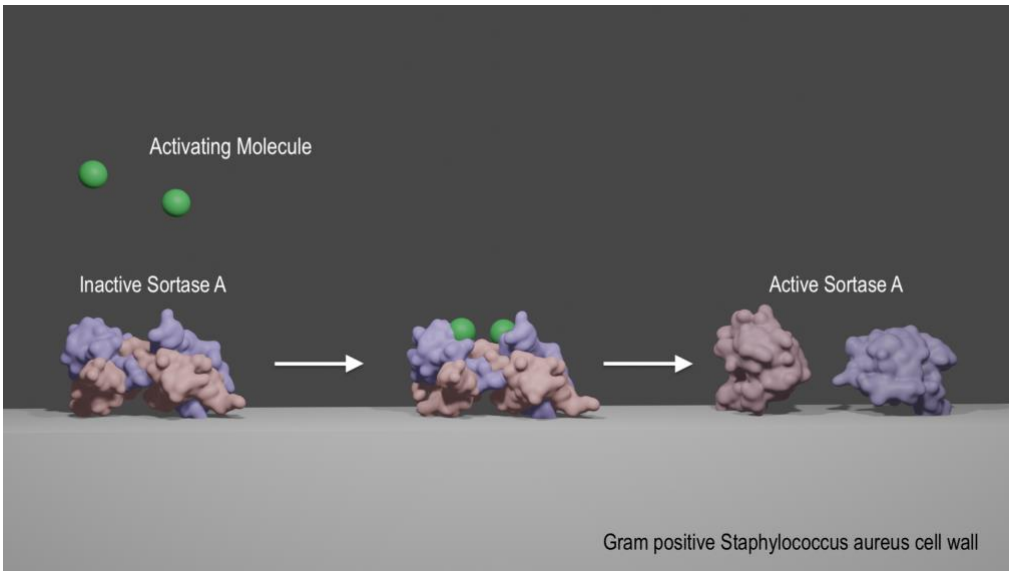

Figure 2. *S. aureus* Sortase A homodimer-monomer equilibrium. © Vivian Zhang

There is no published research on the signaling molecules that interact with Sortase A to regulate the dimer-monomer equilibrium. It's known that bacteria are able to sense various environment cues and respond with alterations of virulence factor gene expression (Jenul, 2019). However, besides bacteria to bacteria communication, bacteria are also able to communicate with host mammalian cells through inter-kingdom signaling. Eukaryotes release small molecules, like hormones, that bind to receptors found on bacteria cells. Binding of mammalian molecules to bacterial receptors initiates a signaling cascade.

Previous research in Dr. Zhang's lab has identified five likely mammalian signaling molecules that interact with Sortase A. The selected signaling molecules could either activate Sortase A to undergo a conformational change, or it can inhibit its conformational change. Our project has been dedicated to testing the efficacy of the signaling molecule as a target for a novel immuno-anti-infective drug.

Protein ID	Protein Name	Function	Location
G3H5I4	NPY-Y2 receptor	Neuropeptide Y receptor	Plasma membrane
G3HTG1	Double C2-like domain-containing protein beta	Calcium dependent phospholipid binding	Plasma membrane
G3IL75	Collagen alpha-1(V) chain	Extracellular matrix structural constituent	Extracellular region
G3HIP9	A disintegrin and metalloproteinase with thrombospondin motifs 12	Metalloendopeptidase activity	Extracellular region
G3IHL7	Cathepsin M	Cysteine-type peptidase activity	Extracellular Region or secreted

Table 1. Five proteins were identified as likely molecules that interact with Sortase A in Zhang Lab.

Overcoming Critiques of Current Approaches

Critiques of Antibiotic Discovery Approaches

The current way of discovering new antibiotic drugs is not efficient. According to WHO, worldwide there are "43 antibiotics in development [:] 15 were in Phase 1 clinical trials, 13 in Phase 2, 13 in Phase 3, and two have had new drug applications submitted" (Hyun, 2021) About one in five drugs can be approved by FDA and enter the clinical trial phase and only 60% of the drugs in Phase 3 clinical trials get approved (Hyun, 2021). The selection process is stringent. Moreover, the average research takes years of work; some even take more than 10 years. The time-consuming part of research is usually due to the screening and testing process of

finding the potential candidates amongst thousands of molecules. This process is time-consuming and expensive due to funding for personnel, lab equipment, maintenance, and cost for consumers in need.

Among other economic factors, these technical and scientific limitations are one of the reasons why it's becoming harder to create new antibiotics that work. This is implicated by a reduced number of FDA approved antibiotics. There were 16 FDA approved antibiotics in 1983-1987 and only 5 FDA approved antibiotics in 2013-2016 (OECD, 2016). These limitations are also why investment into antibiotic research is considered risky and likely unprofitable. There were 18 big pharma companies with an active R&D pipeline in 1990, and this has since reduced to 6 by 2016 (OECD, 2016).

The current Antibiotic discovery approach actively trying to kill the bacteria, by targeting bacteria's cell wall, inhibiting protein synthesis, inhibiting nucleic acid synthesis, and etc.. The targeting molecules of our current antibiotics are molecules that are unique to the bacteria and are not present in human. The antibiotics are biologically safe for human, which allow researchers to target those unique molecules aggressively with the purpose to disrupt the bacteria survival. However, because of bacteria's natural tendency of mutation, this aggressive approach creates survival pressure that encourages bacteria to mutate. With broach application of Antibiotics starting from 1940s, some bacteria survive and gain antibiotic resistance and survive. The first sign of antibiotic resistance happened shortly after the discovery of penicillin: in 1940, an E. coli strain was discovered to produce penicillinase against presence of penicillin (Lobanovska, M., & Pilla, G., 2017). Two years later, the spread of penicillin resistance is documented in four *Staphylococcus aureus (Staph. aureus)* strains in hospitalized patients, and in 1960s, more than 80% of the of the *Staph. aureus* are penicillin resistant (Lobanovska, M., & Pilla, G., 2017). Similar phenomenon of antibiotic resistance was observed when penicillin was introduced into different countries. The repetition of antibiotic resistance occurring and the short timeframe for bacteria to obtain resistance demonstrate the disadvantage of the current antibiotic discovery approach, the evitability of antibiotic resistance.

In the past, scientists can keep up with the speed of bacteria mutation and discover new antibiotics when bacteria become resistance to the current antibiotics. However, more than 150 antibiotics has been discovered and now antibiotic resistance emerges for the majority of the 150 antibiotics. Furthermore, antibiotics abuse aggravates the problem of antibiotic resistance. Some current approaches towards slowing down the antibiotic resistance include reducing antibiotic use in medical practices, improving animal antibiotic use and support the innovations. Antibiotics works against bacteria, but only temporarily. Th approach of competing with bacteria's mutation will not give us a permanent solution to bacterial infection, so we need to look for a new approach that provides longer-term solution to bacterial infection and avoid the problem of antibiotic resistance.

Machine Learning

Our research proposes an efficient approach in antibiotic research by using machine learning to screen for candidates from natural products. Some researchers have shown prominent results using this revolutionary approach: Torres' research focuses on finding naturally existing molecules in the human genome for potential candidates of antibiotics using artificial intelligence. They screened the entire human proteome in search of protein sequences that have antibacterial properties hidden or encrypted within the human genome. "[They] reported the identification of 2,603 encrypted peptide antibiotics that are encoded in proteins with biological function unrelated to the immune system" and elected 55 top candidates (Torres, 2022). The results are not ideal - the candidates showed low effectiveness against bacterial infection. However, they found that a combination of peptides from the same region of infection worked better than a single peptide. This observation is a new pattern that should be taken into consideration when designing or discovering antibiotics. Their approach of using artificial intelligence allows for fast mining of proteomic data, making the search for drug candidates faster and cost-effective compared to the traditional method.

We use machine learning to efficiently find the top candidates for antibiotics of specific bacteria targets. We have the datasets on physicochemical, biological, and functional information on the molecules that can bind to our target, Sortase A. Then, we use these data to train and test the machine learning algorithms. The results should be the best candidate out of thousands of

molecules. We will confirm machine learning results in the wet lab. Besides the basic skeleton structure of the machine learning, we are also experimenting with different standardization methods and models to see if the results are different. In addition, we provide human intelligence to machine learning by adding parameters in addition to the laboratory-obtained information. Based on the machine learning results, some features might be strongly correlated with the "druggability" of the molecules and some features might have little or no correlation (Dezső, 2020). We can manually adjust our algorithm to make it better fit the data based on different research.

Project Statement

Herein, the main goal of our research project is to develop a potential anti-infective drug that *S. aureus* won't develop resistance to by inhibiting an activating molecule of Sortase A. Inhibiting the activating molecule of Sortase A will prevent the expression of virulence proteins necessary for invasion of mammalian cells while also avoiding direct interaction with the bacteria. To overcome the lengthy and costly process of identifying the most potent and safe activating molecule through wet-lab testing, we will utilize bioAI with supervised machine learning as a new approach for drug discovery. We will optimize the bioAI algorithm previously developed by Lisa by incorporating parameters into the machine learning design.

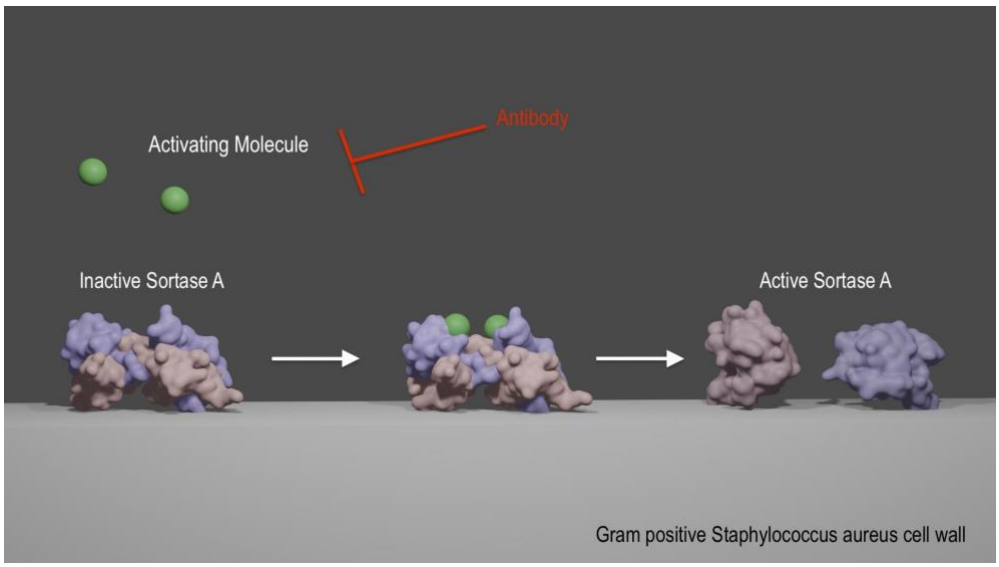

Figure 3. Activating molecule mechanism and antibody development © Vivian Zhang

Significance

MRSA has been an increasingly large issue within the modern healthcare system. “In recent decades, due to the evolution of bacteria and the abuse of antibiotics, the drug resistance of S. aureus has gradually increased, the infection rate of MRSA has increased worldwide, and the clinical anti-infective treatment for MRSA has become more difficult” (Guo, 2020). According to WHO, *S. aureus* infections are of urgent concern, and there’s an urgent need of new antibiotics for methicillin-resistant and vancomycin-intermediate resistant bacteria (Genenva, 2017). If our research works, we can not only discover an anti-infective drug for staph infection, but also propose a new method in drug discovery using bioAI. This method will not only save us time and money for all future drug discovery approaches, but also solve the antibiotic resistance problem worldwide. It would be another advancement in machine learning appliances in the biomedical field beyond bioinformatic. The machine learning technology is evolving constantly. Although similar research is being done on the same topic, the techniques we are using might be different and provide new insights to the field.

Team and Management

Backup Plan

In the event that we are unable to optimize the bioAI algorithm to produce a new list of potential drug targets before the end of Winter Quarter, we have an alternative course of action. Instead of testing drug targets from our own list, we will select the top candidate of the previously selected list of drug targets (Table 1). Unfortunately, for our project, we had to proceed with our backup plan. The selected drug target for our project was NPY-Y2 receptor.

Timeline

During the Fall Quarter, we received training on mammalian cell culturing techniques and planned to conduct the zero experiment. However, our mammalian cells continuously were contaminated with mycoplasma. Therefore, we spent most of our time conducting literature

research into the drug target candidates and practicing wet lab techniques. In the Winter Quarter, we were unable to return to the lab for the first four weeks due to COVID-19 restrictions.

Fall Quarter	Winter Quarter	Spring Quarter
● Finalize materials and experimental plans ● Learn and practice mammalian cell culture techniques	● Invasion assay ● Order mAbs	● Inhibition assay

Table 2. Project timeline in terms of the academic year

Budget

Materials	**Cost**
Cell Solutions (FBS and DMEM)	$226
Mammalian CHO Cells	$710.93
Antibodies	$500
Total Cost	$1,436.93

Table 3. Budget breakdown

Chapter 2: Methods

Our bioAI algorithm will select what it believes to be the most critical activating molecule for Sortase A. Therefore, by inhibiting the activating molecule with its corresponding antibody, it should drastically reduce the invasion of *Staphylococcus aureus* bacteria into mammalian cells. To test the efficacy of inhibiting the activating molecule, we have cultured Chinese Hamster Ovary (CHO) cells to conduct invasion assays. The purpose of the invasion assay is to quantify the level of infection of the CHO cells by *S. aureus*. Three sets of invasion assays were/will be conducted. The zero experiment invasion assay is the negative control where no activating molecule nor antibody were added; therefore, a very minimal level of invasion is expected. The positive control invasion assay will include the activating molecule, but no antibody; therefore, a very high level of invasion is expected. Lastly, the test invasion assay will include both the activating molecule and antibody. The results of this invasion assay will be compared to the negative and positive control to evaluate the effectiveness of targeting the selected activating molecule to prevent bacterial invasion.

BioAI Design

First, I would like to acknowledge the credit of our supervised machine learning algorithm to Lisa Jin. The datasets we are using are collected from external lab and are screened for the binding affinity to Sortase A. Most of the collected parameters are fixed biological parameters such as number or peptide and molecular weight, but the score parameter create some ambiguity. Due to the timeframe when the data was collected, we were not able to trace back the meaning of the score parameter. Along with these parameters, we use the location parameter which are evaluate and weighted by human intelligence (Figure 4). Sortase A interactions occurs at the surface of the cells, so a general scoring guideline would be the closer the molecule is located near the cell membrane the larger the score. However, there're other factors that we took into consideration for molecules that show presences in multiple parts of the cell.

The datasets are preprocessed under feature scaling where we transform each parameter into comparable and standardized data. For parameter values that varies greatly, we would use normalization techniques, and for the parameters with Boolean values, we would transform them

into numerical data. For training and testing of the ML (machine learning) model, 80% of data are used for training and 20% of data are used for testing. The datasets that are used in the machine learning algorithm are less than 3000, which is relatively small. This leads us to the next step ---- wet lab testing, to confirm the results from the ML algorithm.

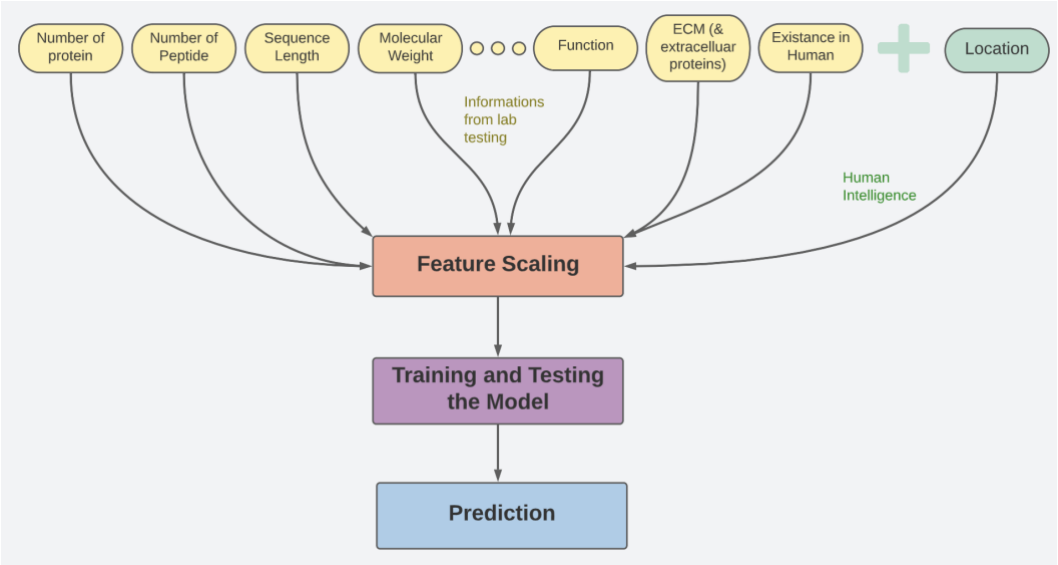

Figure 4. BioAI algorithm structure

Culturing and Passaging CHO Cells

First, we transferred the CHO cells we ordered from stock to two petri dishes. Materials were heated up in a 37°C water bath, including: fresh Dulbecco's modified eagle medium (DMEM), PBS and cell media containing DMEM with 5% Fetal Bovine Serum (FBS). We extracted 2mL of the CHO cell suspension from the stock and put them into a falcon tube, and prepared five falcon tubes containing 2mL of the cell suspension. Then, we spined them in the centrifuge at 3000rpm for 1 minute. A good result should show cell pellet in the bottom of the tubes. After that, we aspirated the liquid in the falcon tubes and avoided suctioning out the cell pellet in the bottom.

The cell pellets observed from 2mL suspension were small, so it’s necessary to combine them before transferring them into the petri dish. So, we combined three of the five falcon tubes into one falcon tube and then the other two into a second tube. Then, 1mL of cell media was added to each tube and the cells were resuspended by pipetting up and down. Petri dishes were prepared

by adding 6mL of cell media, then the cell resuspension solutions were seeded into the petri dish by spreading them in droplets evenly over the surface. Lastly, we transferred the petri dishes into a 37°C incubator.

After we have our CHO cell plates, their condition was checked daily, and media change and cell passage were performed according to their growth. The color of the media is important to note, because oxidation will make the media look brown and oxidation agents are harmful to the cells. When that happened, we changed the media. We also checked for contamination every day, if contamination was spotted, we bleached the petri dishes.

To change the cell media, we first prepared PBS and DMEM with 5% FBS and heated them up to 37°C in the water bath. We first aspirated the old cell media in the petri dish, then washed them with 4mL PBS three times. Lastly, we added new DMEM with 5% FBS to the petri dish.

When cell confluency of 80% or above was observed, we considered passaging the cells into two plates. To passage the cells, we first heated up DMEM with 5% FBS, PBS, and trypsin in a 37°C water bath. Then we aspirated the media in the petri dishes and washed them with 4mL PBS three times. Next, we added 2mL trypsin into the petri dishes and quickly placed the petri dishes into the 37°C incubator for 5 minutes. After 5 minutes, we tapped the petri dishes on the bottom a couple of times to detach more cells. When extracting the cells from the petri dishes, DMEM with 5% FBS was added to the top of the petri dish at an angle to make sure we have all the cells and to neutralize the trypsin. The cell suspension was collected into a 15 mL falcon tube and then spined down in a centrifuge at 3000 rpm for 1 minute. After this step, we should be able to clearly see a cell pellet in the bottom of the falcon tube. Next, we aspirated the supernatant, leaving only the cell pellet in the bottom for resuspension in 2mL of the cell media (DMEM with 5% FBS). Lastly, we prepare the petri dishes by adding 6mL of the cell media to each petri dish, and then pipetting 1mL of cell suspension to each plate by evenly, spread out droplets.

Culturing *Staphylococcus Aureus* (RN4220) Cells

To culture RN4220 bacteria cells, we need the alcohol lamp, LB agar plates, RN4220 bacteria cell stock solution, and inoculation loops. First, we thawed the RN4220 bacteria cell stock, stored in -80ºC condition, at room temperature. Then, we lit the alcohol lamp to create a sterile environment to avoid bacteria contamination. After thawing, we placed the LB agar plate near the alcohol lamp and sterilized the inoculation loop on top of the flame. We dipped the loop into the RN4220 stock solution and then spread the bacteria in the LB agar plate in zig-zag motion. Then, we used parafilm to seal the LB agar plate and placed the plate into the incubator overnight.

To make LB Agar plates, we made a 100mL solution according to the formula that is 7.5g of Agar and 15g of TSB (tryptic soy broth) per 500mL of DI water. Then, we autoclaved the solution in 121°C for 45 minutes. After the solution cooled down, we poured 10 mL of the solution into each plate, and then closed the lid and waited until it solidified. Then, the ten plates were sealed with parafilm, stacked upside down, and stored in a 4°C fridge.

Next day, we inoculated on bacteria in TSB media to allow for more efficient and optimal bacteria growth. The goal for our experiment is to use the bacteria for an invasion assay when it's in the exponential growing phase. To inoculate the bacteria, we first added 5mL of TSB media to a culture tube. Then we used an inoculation loop to pick up one colony from the LB agar plate and dipped in liquid TSB solution. One colony of the bacteria is inoculated into one culture tube. Then, we incubated the bacteria culture in a shaking incubator for five hours under 37°C. After finishing, they are stored in a 4°C fridge waiting to be used for invasion assay.

Invasion Assay

Overall design of the invasion assay experiment is to allow bacteria to invade mammalian cells. This invasion will be conducted in a 6-well plate where three wells contain fluorescent-stained bacteria. The other three wells are the negative control and will not have fluorescent-stained

bacteria. The level of invasion will be quantified using a FACS machine to compare the level of fluorescence between the test and control wells.

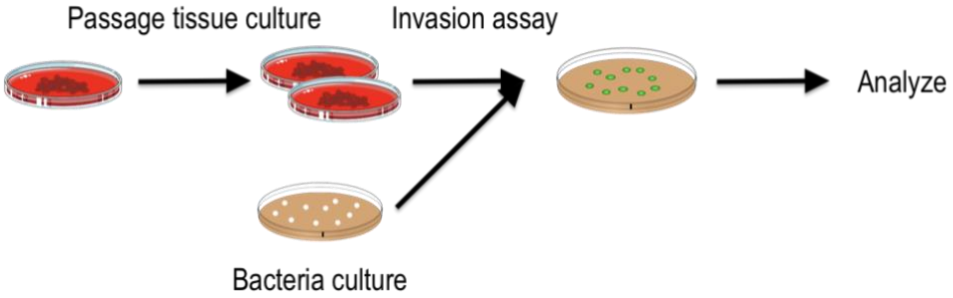

Figure 5. Invasion assay developed by Zhang Lab

Hemocytometer Cell Counting

The day before the invasion assay, CHO cells needed to be passaged into a 6-well plate and counted using a hemocytometer. To do this, two 100mm plates of confluent CHO cells were detached with 2mL of trypsin, incubated for three minutes, neutralized with 5mL of DMEM, and resuspended in 1.5mL Dulbecco's modified eagle medium (DMEM) with 5% fetal bovine serum (FBS). These cells were collected into one 15mL falcon tube to obtain a total of 3mL of CHO cells. 0.5mL of CHO cells were added to each well containing 1mL of DMEM with 5% FBS. Store the 6-well plate overnight at 37ºC and 5% CO2. Next, to approximate the number of CHO cells in each plate, first collect 15µL of CHO cells from the remains of the falcon tube and mix it with 15µL of 0.4% trypan blue solution into a 1.5mL Eppendorf tube. This results in a dilution factor of 2. Add 10µL of the mixed solution into one of the hemocytometer chambers. Count the total number of cells in the four corner squares.

$$Total\ cells/well = \frac{Total\ cell\ count * initial\ volume\ of\ CHO\ cells * dilution\ factor * 10^4}{4}$$

Bacteria Labeling

Bacteria should be in its exponential phase for the invasion assay. Therefore, the day before the invasion assay, one colony was inoculated into four culture tubes containing 5mL TSB media. The culture tubes were then incubated at 37ºC and 220rpm shaking for four hours. After four hours, store the bacteria in 4ºC until it needs to be used for the invasion assay.

On the day of the invasion assay, check the OD600 of the bacteria culture. The desired OD600 is 0.4 to 1.0, which suggests the bacteria are in the exponential phase of growth.

Centrifuge the bacteria culture at 5,000rpm for 10 minutes at 4ºC to obtain a bacterial cell pellet. Remove the supernatant and resuspend the cell pellet with 2mL of PBS. Then, two bacterial cultures were combined into one tube so that there was a total of two culture tubes, each with 4mL of PBS. One of the tubes will be stained with 5mM carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye, and the other will not. To prepare the 5mM CFSE dye stock solution, 18µL of DMSO was added to one vial of CFSE stock tube wrapped in tin foil. CFSE is light sensitive, so it should always be wrapped in tin foil to minimize light exposure. 8µL of CFSE stock solution was added to *one* culture tube. The culture tube stained with CFSE was wrapped in tin foil. Both culture tubes were incubated for 60 minutes in shaker at 225rpm and 37ºC. After 60 minutes, centrifuge the tubes at 5,000rpm for 10 minutes. The CFSE-stained bacteria were then washed with 2mL of PBS three times. Measure the OD600 of the stained and unstained bacteria. The bacteria are now prepared for invasion into CHO cells.

After preparing the bacteria, the CHO cells were prepared for invasion by changing its media with 1mL of fresh DMEM media.

Invasion

The following formula was used to calculate the volume of bacteria cells that needed to be added to each well of CHO cells to achieve a multiplicity of infection (MOI) of 40. The stained and unstained bacteria need to be calculated separately. It was assumed that the OD of 1.0 is equivalent to 8×10^8 cells/mL.

$$Total\ bacteria\ cells/mL = OD600 \times 8 \times 10^8$$

$$Volume\ of\ bacteria\ needed = \frac{Total\ CHO\ cells/well}{Total\ bacteria\ cells/mL}$$

The CHO cells were then mixed with a certain volume of bacteria based on the above calculation. Three wells were mixed with stained bacteria and three wells were mixed with unstained bacteria. The 6-well plate was wrapped in tin foil and incubated at 37ºC for 60 minutes.

After 60 minutes, the bacterial invasion was terminated by adding 6µL of gentamicin to each well and incubating for 45 minutes. After 45 minutes, the CHO cells were washed with 2mL of PBS three times to wash away any debris and bacteria that didn't invade the cells. To analyze the cells, the CHO cells needed to be collected and fixed so that it no longer changes. The CHO cells were collected by adding 1mL of trypsin-EDTA solution to each well, incubating for four minutes, and neutralizing it with 3mL of DMEM. The cells were centrifuged for five minutes at 1340rpm and resuspended with 4% paraformaldehyde (PFA) solution in PBS to fix the cells. The cells were incubated in room temperature for 15 minutes and centrifuged for five minutes at 1340rpm to form a cell pellet. The supernatant was discarded, and the cell pellet was resuspended in 1mL of PBS. The cells were analyzed using a FACS machine.

Inhibition Assay

In the inhibition step, we will add our drug candidate NPY-Y2 receptor antibody (Figure 6) to inhibit the Sortase A from activation, thus inhibiting the bacteria invasion (Figure 7). The inhibition assay is the testing experiment in which we will test the NPY-Y2 antibody's effectiveness in stopping bacterial infection under different concentrations. Procedures for inhibition assay are the same as invasion assay with an additional step of incubating antibiotics for 45 to 60 minutes, before the beginning of the bacteria invasion process.

Candidate NPY-Y2 Antibody

Our final drug candidate, NPY-Y2 (Neuropeptide Y Receptor Y2) Antibodies, is selected from the ML algorithm. NPY-Y2 is the receptor for NPY (Neuropeptide Y), and it can also bind to

Sortase A to active Sortase A, as shown in Figure 6. Our Drug candidate is the NPY-Y2 Antibody, which will bind to the Y2 receptor. NPY-Y2 aims to outcompete the binding between Y2 receptor and Sortase A, preventing Sortase A from binding to the Y2 Receptor, thus inhibiting Sortase A from becoming active. Without active Sortase A, bacteria are unable to invade mammalian cells.

https://www.rndsystems.com/products/human-npy2r-antibody-557521_mab10211

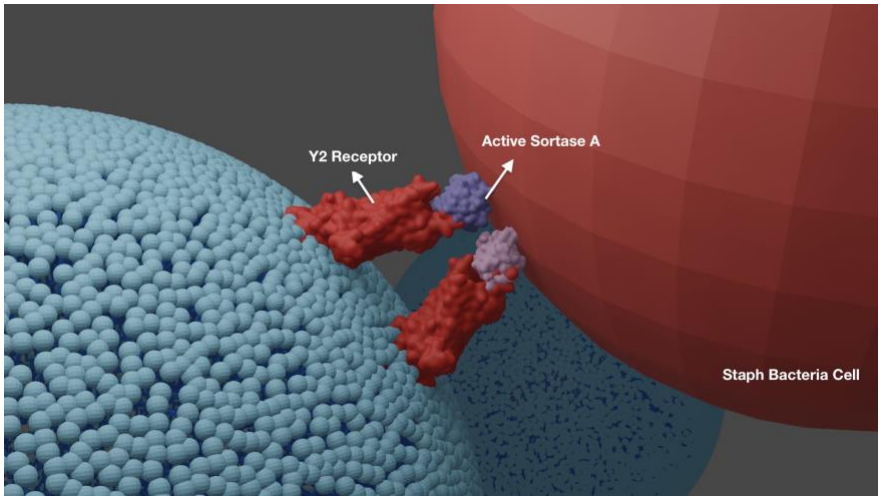

Figure 6. NPY-Y2 mammalian receptor inducing conformational change of *S. aureus* Sortase A from monomer into active dimer conformation © Vivian Zhang

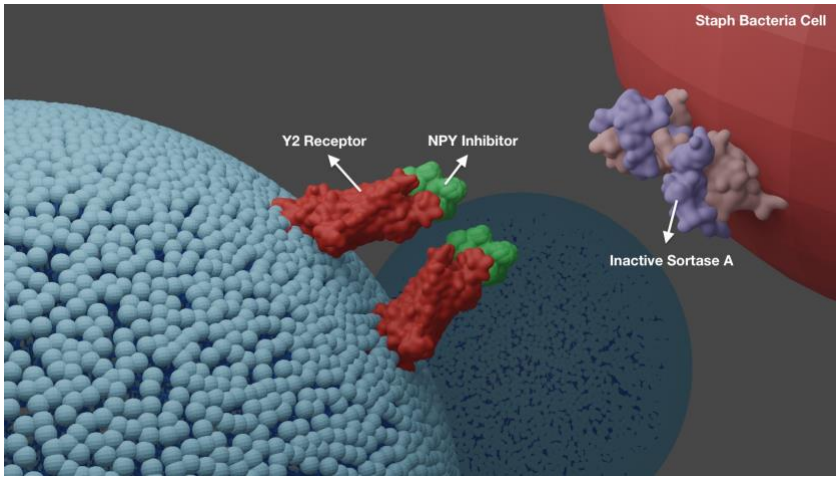

Figure 7. NPY antibody inhibiting NPY-Y2 receptor and preventing activation of Sortase A © Vivian Zhang

CHO Cell Culturing with Antibody Candidate

For each concentration of the NPY-Y2 antibody, we prepare triplicates in 6-well plates. We have in total of 21 wells grouped into 7 groups as following:

A. No antibody (Negative control with unlabeled bacteria)
B. No antibody
C. 0.2 μg/mL
D. 0.5 μg/mL
E. 1 μg/mL
F. 2 μg/mL
G. 8 μg/mL

After the addition of antibody, we culture the CHO cells with antibody for 45 to 60 minutes.

Flow Cytometry

We analyzed the samples using BD Accuri C6 Plus Flow Cytometer. Flow cytometer will collect the samples and measure the cell counts and their corresponding fluorescent level. When using the BD Accuri C6 Plus Flow Cytometer, we need to warm up the machine for 30 minutes. Then we clean the SIP tip, where the samples are being collected, and run the instrument QC (quality control) to ensure that the flow cytometer can function properly. SIP clean is performed with SIP clean liquid and DI water. Then, we can insert the samples in the SIP tip to collect data. SIP clean is performed, between data collection of each sample as well. Figure 8 indicates the mechanism that infections from CFSE-stained bacteria can make CHO cell fluorescence.

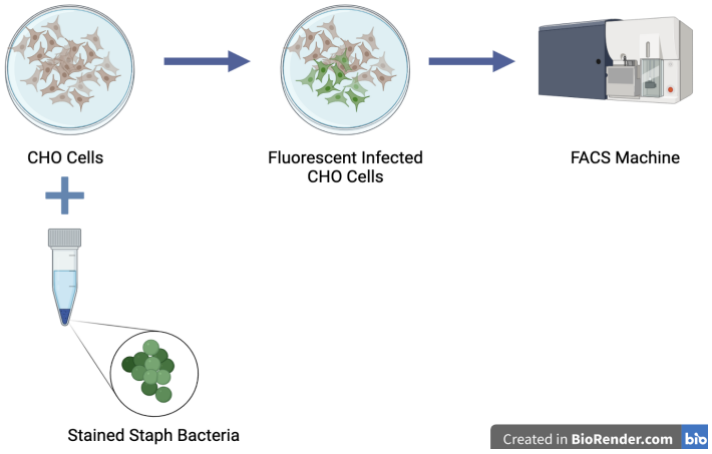

Figure 8. Invasion and inhibition assay analysis using flow cytometry

Chapter 3: Data Analysis and Results

Flow Cytometry

The degree of internalization, or invasion, of *S. aureus* into the CHO cells was measured using the BD Accuri™ C6 Plus Software flow cytometry. The flow cytometer counts the number of cells it collects and measures each cell's fluorescence level. Therefore, by staining the bacteria with CFSE dye, we can analyze the degree of internalization into mammalian cells based on the relative fluorescence intensity. The higher the degree of internalization, the higher the fluorescent intensity. The flow cytometer measures fluorescence level using fluorescein isothiocyanate (FITC)-A mean fluorescence intensity. The FITC-A score is plotted against cell count and the gating was manually adjusted based on the general peaks of the graph representing CFSE-labeled and unlabeled bacteria trials.

The uptake index measures the relative degree of internalization based on the fluorescence level of the CHO cells. It allows for comparison of data between experiments by taking into account the relative proportional differences of number of CHO cells to bacteria cells. Therefore, the uptake index also suggests the activity of Sortase A, which allows for invasion of *S. aureus* in the CHO cells.

*Uptake Index (UI) = Mean FITC-A * Percentage of Fluorescent Molecules*

Invasion Assay

We performed the invasion assay four times throughout the school year, and we were finally able to complete a successful experiment in our fourth experiment. For the invasion assay, three wells represented the negative control which had CHO cells infected with unlabeled bacteria. And another three wells represented the positive control which had CHO cells infected with CFSE-labeled bacteria.

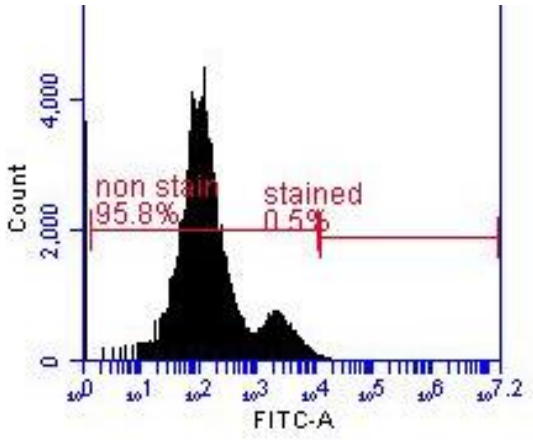

Figure 9. Negative control (unlabeled bacteria). The x-axis, FITC-A, represents the fluorescent level from low to high. The y-axis represents the cell count.

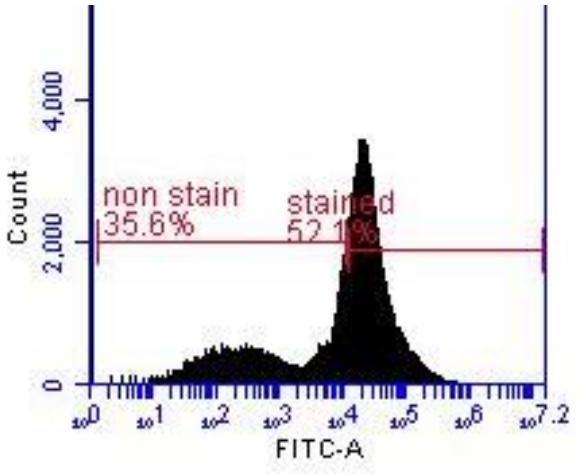

Figure 10. Positive control (CFSE-labeled bacteria). The x-axis, FITC-A, represents the fluorescent level from low to high. The y-axis represents the cell count.

		Raw Data		
Antibody Concentration (µg/mL)	**Wells**	**Mean FITC-A**	**FITC-A Stained Percentage**	**Percentage of area (%)**
0 (Negative Control, unstained)	A1	565.68	73.24	0.46
	A2	3,387.42	40.91	5.14
	A3	2,676.00	35.55	0.82
0 (Positive Control)	B1	54,971.66	306.7	52.07
	B2	141,801.80	145.68	51.88
	B3	53,722.23	307.38	51.98

Table 4. Raw data collected from flow cytometry of invasion assay

		Processed Data		
Antibody Concentration (µg/mL)	**Wells**	**Uptake Index (UI)**	**Average UI**	**Normalization**
0 (Negative Control, unstained)	A1	414.304032	917.138518	0.005092325188
	A2	1385.793522		
	A3	951.318		
0 (Positive Control)	B1	168598.0812	180102.1113	1
	B2	206576.8622		
	B3	165131.3906		

Table 5. Processed data with UI and error bar calculations

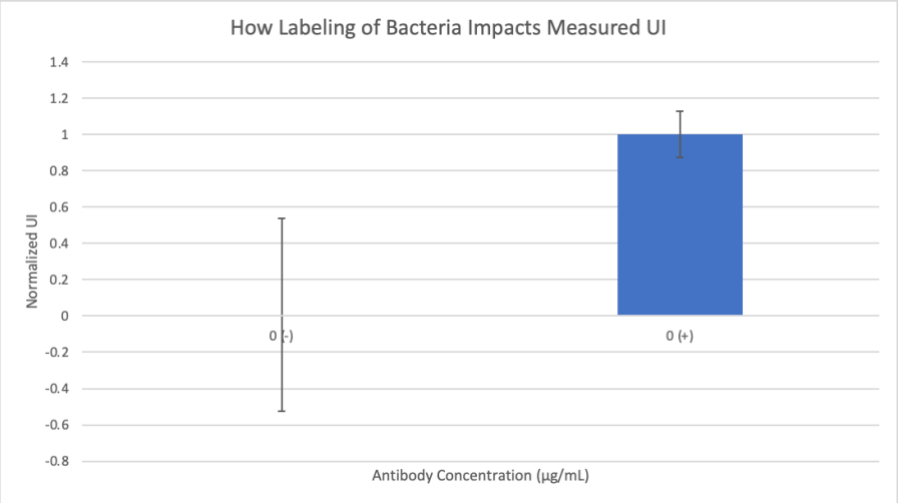

Figure 11. Final results of invasion assay

Inhibition Assay

For the inhibition assay, we conducted 7 trials of varying antibody concentrations in triplicate. All wells were infected with either CFSE-labeled or unlabeled (negative control) bacteria.

		Raw Data		
Antibody Concentration (µg/mL)	**Wells**	**Mean FITC-A**	**FITC-A Stained Percentage**	**Percentage of area (%)**
0 (Positive Control)	A1	35,127.55	91	6.5
	A2	33,065.98	84.9	5.1
	A3	19,739.84	87.4	3.2
0.2	B1	39,460.67	84.6	9.8
	B2	32,917.81	73.3	7.5
	B3	13,630.06	85.7	0.70
0.5	C1	36,869.85	14	0.4
	C2	22,170.89	77.5	3.2
	C3	29,922.30	80.7	8.7
1	D1	42,151.69	72.4	12.4
	D2	16,870.77	82.2	2.5

	D3	70,524.21	82.6	32.1
2	E1	16,798.42	91.7	2
	E2	53,153.67	85.4	16.6
	E3	58,144.34	83.3	28.6
8	F1	15,782.05	87.7	3.1
	F2	19,293.13	82	3
	F3	15,150.84	73.4	2.4
0 (Negative Control, unstained)	G1	6,982.30	9.4	3.6
	G2	6,576.16	53.6	23.7
	G3	3,636.97	8	3.1

Table 6. Raw data collected from flow cytometry of inhibition assay. Red highlighted trials (C1 and G2) are outliers that were not included in data processing.

		Processed Data		
Antibody Concentration (µg/mL)	**Wells**	**Uptake Index (UI)**	**Average UI**	**Normalization**
0 (Positive Control)	A1	31966.0705	25763.90256	1
	A2	28073.01702		
	A3	17252.62016		
0.2	B1	33383.72682	23064.48099	0.8952246631
	B2	24128.75473		
	B3	11680.96142		
0.5	C1	5161.779	20664.86793	0.8020860922
	C2	17182.43975		

	C3	24147.2961		
1	D1	30517.82356		
	D2	13867.77294		
	D3	58252.99746	34212.86465	1.327937977
2	E1	15404.15114		
	E2	45393.23418		
	E3	48434.23522	36410.54018	1.413238545
8	F1	13840.85785		
	F2	15820.3666		
	F3	11120.71656	13593.98034	0.5276366927
0 (Negative Control, unstained)	G1	656.3362		
	G2	3524.82176		
	G3	290.9576	473.6469	0.01838412868

Table 7. Processed data of inhibition assay. Red highlighted trials (C1 and G2) are outliers that were not included in Average UI calculations.

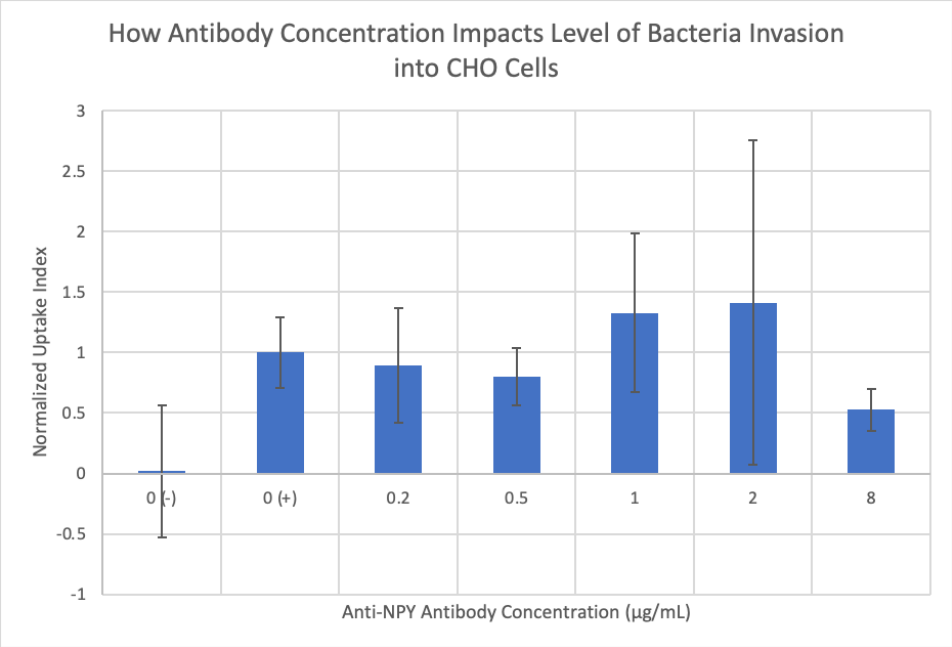

Figure 12. Final results of inhibition assay

Chapter 4: Discussion and Conclusion

Invasion Assay

The invasion assay needed to be repeated several times due to technical error during the invasion assay experiment (e.g. cross-contamination of samples) and technical difficulties with the flow cytometry. Once we resolved our own technique issues, we tried to overcome difficulties with the flow cytometry by analyzing our samples using a fluorometer to measure fluorescence intensity and hemocytometer to count the remaining cells. Unfortunately, we still obtained unusual results that were not reliable and could not be processed to measure the uptake index. The results were unreliable because the DI water showed fluorescence as well. Fortunately, in our fourth experiment, the flow cytometer was fixed, and we were able to process our data on the day of the experiment.

For the negative control, ideally we expect to see 100% of CHO cells to be unlabeled because the flow cytometry should count all CHO cells and measure minimal fluorescence in all of them. As shown in Figure 9, in this negative control well, 95.8% of the collected CHO cells were unlabeled. This is shown by a significant peak at a low FITC-A score. This result is as expected for the negative control. This suggests that there was no cross-contamination of the negative control with the CFSE-labeled bacteria in the positive control wells.

For the positive control, we expected to see two peaks of CHO cells infected with labeled and unlabeled bacteria. As shown in Figure 10, in this positive control well, 52.1% of the CHO cells were infected with labeled bacteria.

After measuring the normalized uptake index and calculating the standard deviation for the error bars, the final results of the invasion assay in Figure 11 shows a significant difference between the CHO cells infected with labeled and unlabeled bacteria. The invasion assay experiment suggests that the CFSE-labeling of bacteria can be an effective way to evaluate the relative degree of internalization. We can now proceed with the inhibition assay using this technique.

Inhibition Assay

This was our first time conducting the inhibition assay. As shown in Figure 12, the error bars are significant. This suggests a low precision and high variability of data collection. Therefore, the data collected is not reliable and needs to be repeated. Nonetheless, the data produced interesting results that are worth mentioning and exploring.

For the inhibition assay, we expected to see a decrease in uptake index as the antibody concentration increases. As shown in Figure 12, there is the expected decrease in uptake index; however, at higher antibody concentrations of 1μg/mL and 2μg/mL, there's an increase in uptake index. Although this upward trend at higher antibody concentrations could be due to technical errors during the experiment, this trend could also illustrate the CHO cell's temporal resistance to the NPY-Y2 antibody drug.

It's known that cancer cells have the ability to adapt and become resistant to immunotherapies similar to how bacteria cells develop resistance to antibiotics (Farquhar, et. al., 2019). The trend seen in Figure 12 is also seen in recent research studies exploring drug-dependent or stress-dependent growth curves for multicellular organisms like bacteria, mammalian cells, and cancer cells (Guinn, et. al, 2022). This adaptation, unlike resistance acquired from genetic mutations, is temporal and correlated with drug concentrations. The adaptation can occur within hours and is likely due to an intrinsic stress response (Guinn, et. al, 2022).

Furthermore, previous research explores the stochastic effect that explains why genetically identical cells may differ drastically when exposed to the same stimuli. The stochastic effect, in contrast to the deterministic effect, refers to the statistical, dosage-independent nature of a stimuli like a drug (Farquhar, et. al., 2019). In other words, the dosage of a drug doesn't determine the effect on a mammalian cell; instead, a higher dosage only increases the *probability* of an effect. Numerous examples of the stochastic effect have been highlighted in research, even on the single-cell level where gene expression fluctuates stochastically (or gene expression noise). Gene expression noise likely occurs due to the "intrinsic randomness of underlying biochemical reactions or processes extrinsic to the gene" (Farquhar, et. al., 2019). This response is regulated by an intrinsic stress response. It's currently unclear how gene expression noise

impacts drug resistance in mammalian cells. However, understanding the development of mammalian cell resistance to drugs could have profound implications on cancer immunotherapy as well as our research on immuno-anti-infectives. Furthermore, this could also make clear pharmacodynamics and drug dosing.

Future Steps

Inhibition Assay

More inhibition assay trials need to be done to produce more reliable results for better interpretation. Research has shown that an incubation time of around 1-2 hours is recommended for the murine IgG1 antibody; however, it's recommended to conduct our own trials of varying incubation time to assess its impact on the uptake index. Another adjustment to the inhibition assay includes reducing the range of antibody concentration to be 0 - 4 µg/mL instead of 0 - 8 µg/mL. This will also help elucidate the potential drug-dependent growth curve discussed earlier. Furthermore, another possible experiment is to assess the inhibition assay using neural cells. Neural cells have the highest expression of NPY-Y2 receptors (Hökfelt, et. al., 1998). Therefore, by testing the antibody's effectiveness, we can find a more accurate correlation between concentration and invasion rate.

BioAI Optimization

Currently, the BioAI algorithm only selects drug target candidates based on the location of the drug target. Additionally, parameters need to be added to optimize the selection of potential drug target candidates. One set of parameters include physicochemical features of the protein (e.g. estimated half-life, isoelectric point) which ultimately determines the protein's function and chemistry. Another set includes structural features (e.g. types of chains, cross-links, β-strands, turns, helices, transmembrane domains) which determine the protein's binding abilities. And lastly, functional features (e.g. subcellular location, interaction with different proteins, metal-binding) which provides insight into the protein's involvement with different pathways and potential diseases. The parameters need to be selected while taking into account the significance or impact of the parameter on the safety and efficacy of the anti-immuno-infective drug design.

Some parameters can be obtained from public databases, while other parameters would require further laboratory tests. More parameters involving human intelligence that give scores based on researcher's knowledge would also be considered. In the end, the purpose of using BioAI is to accelerate research's progress under supervision. Another aspect for future improvement is to add more drug candidates to our current datasets. More training and testing data will allow the ML algorithm to have better accuracy. In our project, our dataset is screened for binding affinity with Sortase A, narrowing down the options. However, this strategy infringes on the validity and accuracy of the ML algorithm itself. BioAI has the potential to accelerate and aid in drug research and discovery. With more data input, we are hoping that our model can be improved, and similar strategies can be applied to anti-infective drug research in general.

Chapter 5: Engineering Standards and Realistic Constraints

Ethical

The Ethics aspect of our project would be the choice of cell line. We use the CHO (Chinese hamster ovarian) cell line. Because it's an animal cell line, it's less controversial than a human cell line.

Social

Our project aims to find a drug that can prevent staph. bacterial infections in a clinical setting which would decrease the mortality caused by bacterial infection. Also, if our approach of incorporating machine learning in drug development would be applied to other research of anti-infective drugs, there will be a more efficient way for anti-infective drug research and development.

Political

Excessive use of antibiotics often correlates with inappropriate prescription and administration and antibiotics therapy. Research has found that there's a positive correlation between antibiotic usage and antibiotic resistance where in areas that consume higher amounts of antibiotics, higher rates of antibiotic resistance are found (Lobanovska, M., & Pilla, G., 2017).

Antibiotics are also being widely abused in the agricultural industry. Antibiotics are commonly used to prevent infections and as growth promoters in livestock. In many developed nations, livestock consume an estimated 50–80% of antibiotics (Cully, 2014). Humans consume most of the rest, with crops, pets and aquaculture collectively accounting for about 5% (Cully, 2014). This is not only related politically, but also tied into environmental and health aspects.

Environmental

This abuse of antibiotics in the agricultural industry leads to pollution to the environment. The antibiotic resistant gene can increase by up to 28,000-fold in soils in one year (Wang, etc.…, 2022). This pollution also influences the water sources, along with the organisms that live in water.

Health and Safety

With antibiotic-caused pollution from agriculture, we can also see higher risks of interspecies viral transmission, as well as the spread of antibiotics resistance genes from bacteria that infect animals to bacteria that infect humans, escalating the problem of antibiotic resistance in bacteria.

From a drug design perspective, we will carefully consider the trade-offs that determine which molecules will be the most suitable option for drug design. The drug will target molecules that originally exist in our body that can also activate Sortase A. Our drug will be designed to inhibit the molecules playing a certain role in the human body. We may or may not know the exact outcome of inhibiting the target molecules. So, we need to consider if the human body can endure these side effects. If the side effects are not severe, we need to take careful consideration and thorough testing to determine the safe dosage one would use.

Sustainability

The sustainability of our project is mostly focused on lab supplies and minimizing the waste produced by protocol optimization.

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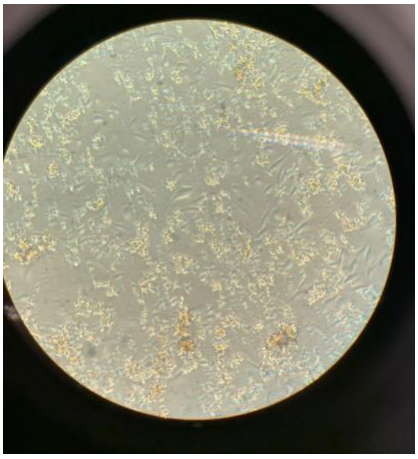
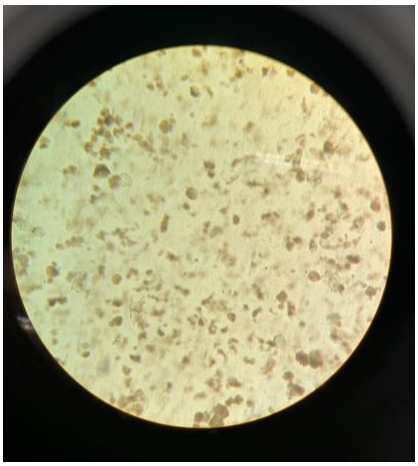
Appendices

Appendix A: CHO cells contaminated with mycoplasma (A), with bacteria (B), and healthy CHO cells (C)

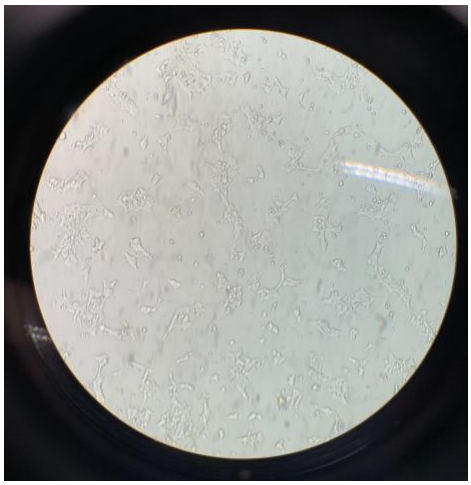
A.



B.

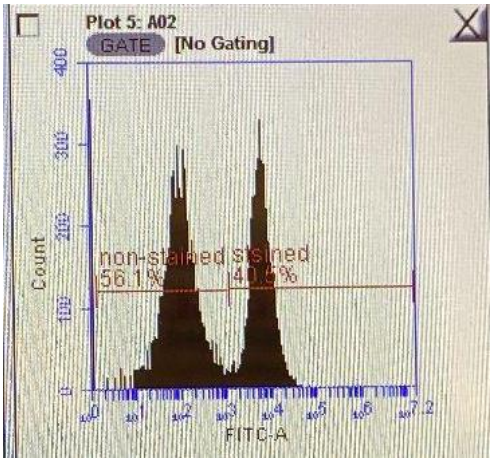


C.



Appendix B: Invalid invasion assay data results. A) Negative control shows cross contamination with CFSE-labeled bacteria trials from flow cytometry analysis. B) Fluorometer analysis shows fluorescence for DI water, suggesting improper calibration of fluorometer settings.

A.



B.