Design Brief

Developing new aptamers to combat antibiotic resistance by screening aptamer libraries and a proof of principle microbicidal aptamer that selectively binds to an Ampicillin-binding protein*

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Antibiotics have changed the course of medicine in the twentieth century, significantly reducing mortality from infectious diseases across the world. However, as more antibiotics are used, their effectiveness wanes due to resistance developed by microbes. Creating and screening new antibiotics is a costly and time-consuming process with diminishing returns on investment, today's antibiotic development pipeline is reported to be weak and the rate of which it is happening does not match bacteria's increasing ability to mutate and develop resistance. To solve this problem, aptamers can be used to mimic different classes of antibiotics. Aptamers are sequences of DNA or RNA molecules that can fold in various configurations and selectively bind to targets including proteins, peptides, carbohydrates, and toxins. For example, we can screen for an aptamer that can bind to the target of a particular antibiotic and therefore it can be used to act like an antibiotic. Unlike antibiotics, aptamers can be produced more efficiently and at a much lower cost. Since aptamers can be easily developed in the lab, if bacteria develop resistance to an aptamer, a new aptamer can be developed quickly to overcome the resistance, unlike antibiotics. Because sptamers are short-lived in the body, they have lesser side effects and are less likely to promote resistance development in microbes compared to antiobiotics. Also, they are short-live in the environment, thus contributing less to environmental pollution. This project aims to set up a microbicidal aptamer screening method. The prototype aptamer will mimic a common laboratory antibiotic, ampicillin, and bind to its target, which is the peptidoglycan transpeptidase enzyme found in bacteria. Once a proof of concept is established for ampicillin, the screening method can be adopted for additional antibiotics and against various microbial molecular targets.

Keywords: Aptamer, antibiotic, antibiotic resistance, ampicillin

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The increasing availability of antibiotics globally has led to their overuse and the emergence of antibiotic-resistant strains. According to the Centers for Disease Control (CDC) and the World Health Organization (WHO), antibiotic or antimicrobial resistance is a global public health threat. The CDC website cites that antibiotic resistance was associated with nearly 5 million deaths worldwide in 2019. *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa* are responsible for 80% of the 1.27 million deaths that were caused directly by antimicrobial resistance in 2019 (Murray, CJL, et. al., 2022).

Antibiotics are used to inhibit growth, block vital processes, and eventually kill bacterial cells to stop them from spreading in the body (“About Antibiotics.”). Although there are many types of antibiotics, one of the most common types is beta-lactam antibiotics, which include penicillin and ampicillin. Beta-lactams can be further divided into broad-spectrum antibiotics, which can target a wide range of bacteria, and narrow-spectrum antibiotics, which only target a small range of bacteria. All beta-lactam antibiotics work by inhibiting the formation of the bacterial cell wall, more specifically a component of the cell wall called the peptidoglycan. This is done by the antibiotic binding to and inhibiting penicillin-binding proteins (PBPs) which are involved in building the peptidoglycan in both gram-negative and gram-positive bacteria. One of such PBPs is a transpeptidase enzyme that is involved in the final stages of building peptidoglycan. Without proper peptidoglycan formation, the cell wall is severely weakened, and bacteria eventually be lysed and die because of pressure imbalances between the inside and outside of the cell.

Antimicrobial-resistant infections require the use of second and third-line treatments that can have serious side effects such as organ failure and patients typically require prolonged care and recovery. The current mitigation strategies for the spread of antibiotic resistance are to regulate the use of antimicrobial compounds and prevent the spread of resistant bacteria if an infection does happen. Educating nurses, physicians and other healthcare providers about the antimicrobial resistance crisis and practices that can help combat the spread of infection is another vital component of the current strategy (Uchil et al., 2014). Antibiotic resistance develops when bacteria are exposed to low levels of antibiotics for long periods, causing bacteria to develop immunity to the antibiotics that were designed to kill them (Arsène, M, et al, 2022).

To address this issue, I propose to use aptamers as a novel antimicrobial molecule. Aptamers are stable DNA or RNA molecules that bind with specificity to targets such as small molecules, peptides, proteins, cells, and tissues with high affinity. In this proof-of-concept study, s I will design, screen, and characterize aptamers that mimic ampicillin, a common laboratory beta-lactam antibiotic. Aptamers have been accepted as therapeutics for human use, having been used in clinical trials and approved by the FDA to treat macular degeneration (Ni et al., 2011).

Systems level

A visual representation of how an aptamer can bind to a target is shown in Figure 1. Figure 1A shows the conformation of a free aptamer and Figure 1B shows induced fit binding between aptamer (orange and blue) and its target protein (yellow). Residues of the protein that are interacting and binding to the adaptmer are in red. Using this concept, my project aims to create an aptamer that mimics ampicillin and targets the peptidoglycan transpeptidase protein.

In bacteria, the peptidoglycan transpeptidase enzyme is an excellent drug target because it is essential for bacteria survival, accessible from the periplasm, and

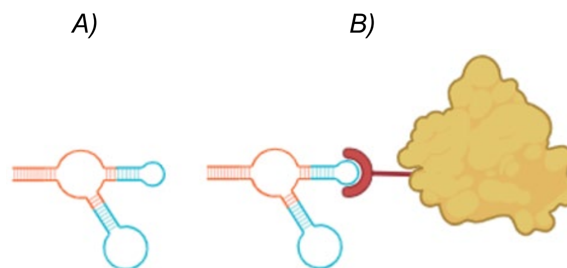

Figure 1. How an aptamer binds to its target.

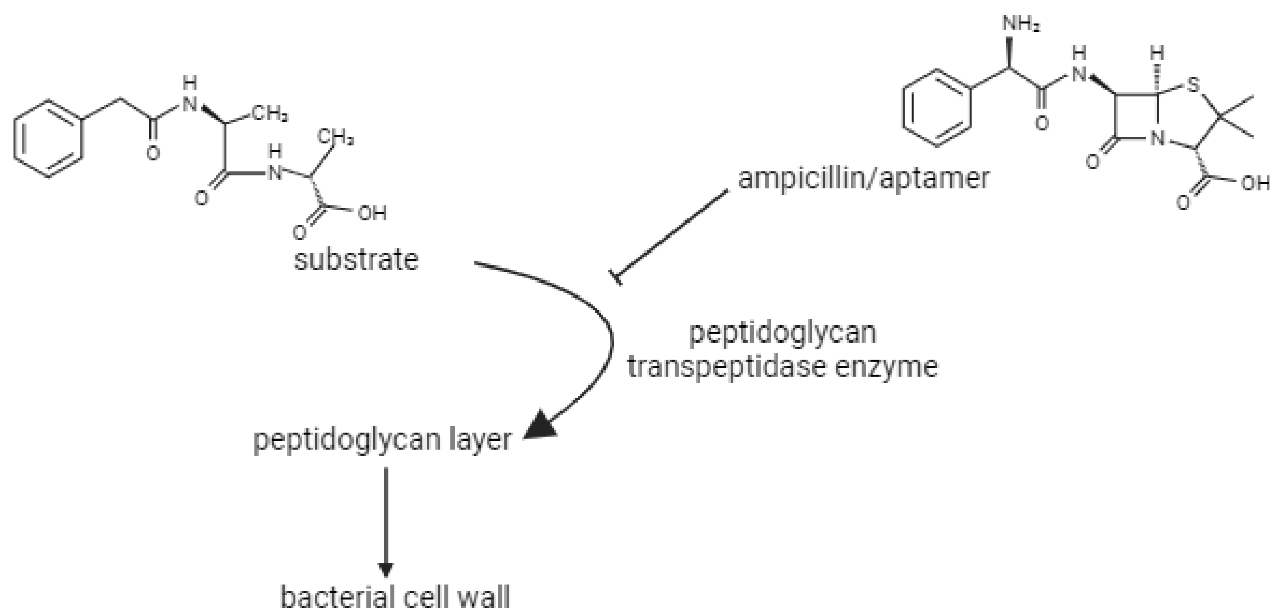

Figure 2. Structural similarities of the aptamer or ampicillin to the substrate allows it to bind the peptidoglycan transpeptidase and inhibit the production of the bacterial cell wall.

has no equivalent in human cells thereby reducing risk of off-target effects. It is the target protein of ampicillin because the structure of ampicillin closely resembles the D-Ala-D-Ala residue of peptidoglycan, the natural substrate of the peptidoglycan transpeptidase enzyme, as shown in Figure 2. This enzyme helps in the synthesis of peptidoglycan which is a component of the bacterial cell wall. In the absence of a cell wall, the bacterial cell membrane will dissolve or be lysed, killing the bacteria.

Device level

My proposed aptamer will bind the active site of the enzyme irreversibly, similar to the mode of action of ampicillin, resulting in the inactivation of the enzyme. To do so, I will implement the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology (Figure 3). SELEX is a method that allows for the systematic screening of ligands or the aptamers that bind the target of choice. The aptamers enriched in the first screening are re-screened in a second round to find aptamers that can bind more effectively to the target. This process is repeated several times resulting in the step-wise enrichment of the aptamer with the highest affinity to the target.

Currently, SELEX is the technology of choice to work with aptamers due to its ease and efficiency. The SELEX technology will be used to screen for aptamers that are able to bind to the peptidoglycan transpeptidase enzyme specifically, drastically reducing the amount of time and cost required to find the correct aptamer. A commercially available aptamer library will be used for the experiment. The experimental steps are outlined in the section below.

Parts level

A typical aptamer library contains between 10^4 and 10^5 different random sequences and the selection process, illustrated in Figure 3, can occur anywhere between 6 and 15 times to identify aptamers with the highest affinity and specificity (Kong & Byun, 2013).

As illustrated in Figure 3 above, the following steps will be performed

1. Clone, express, and purify the active site of the peptidoglycan transpeptidase enzyme
   a. We will clone the active site in a bacterial expression vector with a

histidine tag. The fusion protein containing a histidine tag and the active site of the peptidoglycan transpeptidase enzyme will be expressed in *E. coli*. The fusion protein will then be purified using a histidine binding column.
   b. In order to avoid nonspecific binding of aptamers, which can lead to inaccurate and inefficient aptamer selection, only the active site will be cloned and used in this experiment.
2. Prepare a nickel column and bind the protein that was cloned in Step 1 to the column.
3. Pass the library of aptamers over the nickel column and wash away the unbound aptamers
4. Elute the bound aptamers to obtain a fraction of the library enriched in desirable candidates.
5. Amplify the bound aptamers (first set of enriched library) using polymerase chain reaction (PCR)
6. Repeat the selection process (SELEX) on the enriched aptamer population for 6-15 rounds to obtain the final candidate aptamer with the highest binding specificity and selectivity to the target.
7. Sequence the final candidate aptamer to identify the aptamer
8. The antibacterial activity of the selected candidate aptamers will be checked by growth inhibition of suitable laboratory strains of *Escherichia coli*. Some of the data we would collect throughout the experiment would be aptamer sequences and tertiary structures of aptamers. We would also measure binding affinity to the target protein, affinity constants, and inhibition of bacterial growth. Specifically, we would measure the optimal dose of the aptamer inhibition of bacterial growth. Ampicillin can enter the

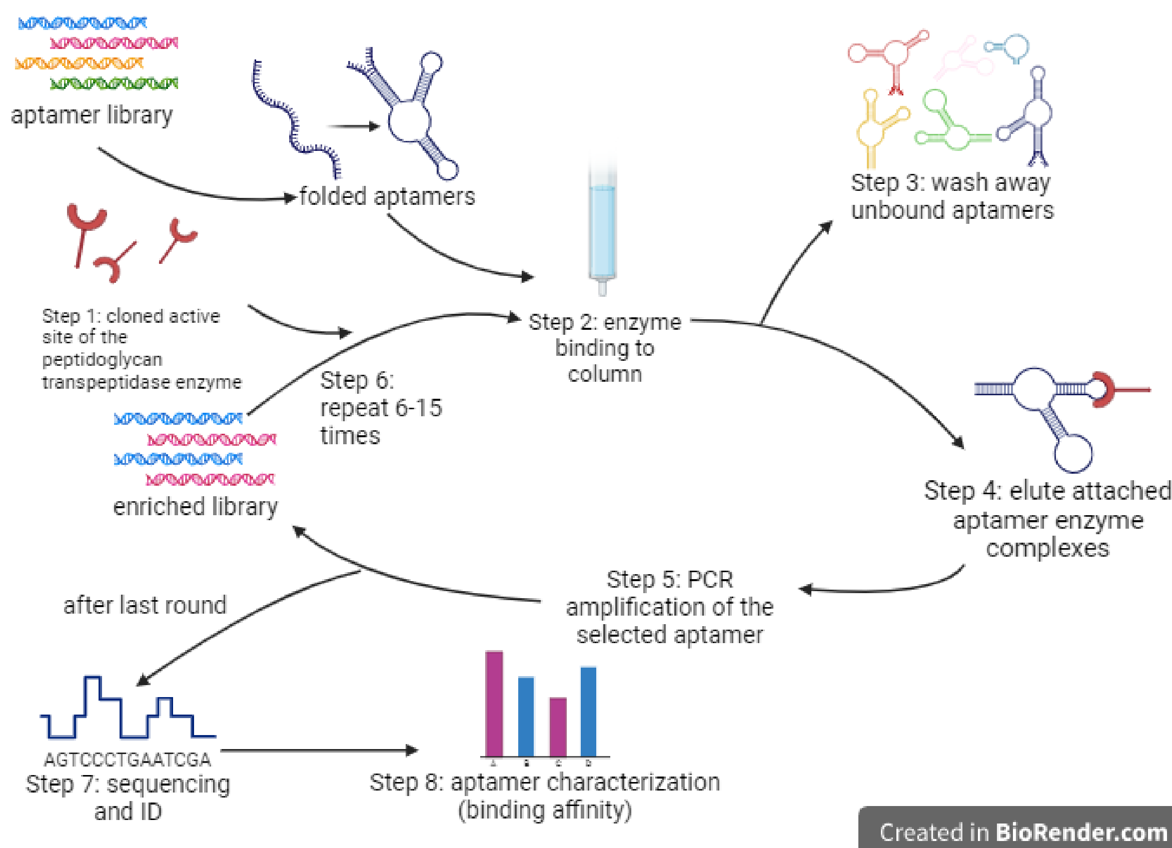

Figure 3. Diagram of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process to select the correct aptamer. The target molecule will be an ampicillin resistant protein.

bacterial cell and gain access to the target, peptidoglycan transpeptidase enzyme. Based on literature, aptamers can also enter the cell through endocytosis (Yoon and Rossi, 2018).

However, if we find that the selected aptamer is unable to enter the bacterial cell, we can perform the SELEX screening on the bacterial cells (as targets) rather than the enzyme itself.

Safety

The experiments described in this proposal require basic equipment that is widely available in a biology laboratory. The use of the equipment and working in the laboratory will require training on general laboratory safety protocols. The chemicals and supplies used will be disposed of following the laboratory practices and to meet the local safety and hazardous material disposal requirements.

The only living organism used in a later part of the study will be a non-pathogenic laboratory strain of *E. coli* that will be killed and disposed of after the experiments following the laboratory practices and safety rules.

Appropriate personal protective equipment, such as lab coats, gloves and safety glasses will be used. The laboratory areas used will be cleaned and disinfected following laboratory rules.

Discussions

In order to find the appropriate aptamers that we can use to imitate antibiotics, several advancements and breakthroughs are necessary. Currently, this proposed experiment is a preliminary proof-of-concept for screening antimicrobial aptamers. If this project is successful, our next step would be to see if one of these aptamers can functionally inhibit the growth of or kill ampicillin-resistant bacterial strains. Another important advancement is that we would have to figure out how to improve the properties, such as stability and clearance rate of the aptamer in the human body. Most notably, a breakthrough necessary to proceed with the experiment is to go through the SELEX process specifically for the peptidoglycan transpeptidase enzyme. This selection needs to be done several times so that we can find an aptamer with the highest affinity and specificity to the enzyme.

One of the inherent challenges of using aptamers instead of antibiotics, like any new technology, is that it will take time for widespread application and the aptamer would have to undergo many rounds of clinical trials.

The idea of using aptamers as therapeutic drugs is now in clinical trials and has proven to be safe (Nimjee et al. 2017). If we face challenges during clinical trials for any reason and must go back to the lab to improve the aptamer for efficacy or stability in the body.

In summary, based on the approach described here, the time and cost required to design and manufacture novel aptamers is far less than that required to discover and manufacture novel antibiotics. Therefore, aptamers can be utilized to help us combat the challenge of antibiotic resistance.

Next steps

If the initial project with ampicillin is successful, the screening method can then be adopted to screen for antibiotics for various bacteria, including antibiotic-resistant bacteria, and against various microbial molecular targets. Using a similar approach for another antibiotic, we would create a new aptamer that binds to the active site of another antibiotic. An aptamer library would be used to select different aptamers that are suitable for this antibiotic and it would undergo SELEX to obtain ideal candidate aptamers with high binding specificity and selectivity. If the correct aptamers are found, it would then undergo many rounds of clinical trials before being used for the general public to treat bacterial infections.

Author contributions

I researched and wrote this paper individually, communicating with other

members of the BioBuilders Club at Andover High School when necessary. They provided help with the logistics and organization of the paper and gave me constant feedback on how to improve.

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