ABSTRACT

Inhibition of β-lactamase I from *Bacillus cereus* by ssDNA

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Inhibitors of β-lactamases are important to the treatment of infectious diseases when used in conjunction with a β-lactam antibiotic. Current inhibitors of β-lactamase such as clavulanic acid, sulbactam, and tazobactam perform efficiently overall but due to developing bacterial resistances to these inhibitors, new inhibitors need to be discovered. SELEX procedures were used to isolate ssDNA aptamers capable of binding to the enzyme active site and consequently inhibit the action of β-lactamase I from *Bacillus cereus* 569/H/9. A 22 base ssDNA aptamer was discovered to have an inhibition pattern consistent with reversible competitive inhibition. These results prompted further study of a hairpin loop of 10 bases and a linear 11 base ssDNA aptamer which were truncated forms of the original 22 base aptamer. The 11 base aptamer failed to show any inhibition against β-lactamase I, whereas the 10 base aptamer showed competitive reversible inhibition.

Inhibition of β-lactamase I from *Bacillus cereus* by ssDNA

by

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A Thesis

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LIST OF ABBREVIATIONS

B. cereus ..... *Bacillus cereus*

BSA ..... bovine serum albumin

DNA ..... deoxyribonucleic acid

dsDNA ..... double stranded deoxyribonucleic acid

E. coli ..... *Escherichia coli*

IC_{50} ..... inhibitor concentration at 50 % inhibition

K_i ..... dissociation constant for inhibitor from enzyme-inhibitor complex

K_m ..... the Michaelis-Menten constant

LB ..... Luria-Bertani

MIC ..... minimum inhibitory concentration

MIC_{90} ..... minimum inhibitory concentration 90% inhibition

MOPS ..... 3-(N-morpholino) propanesulfonic acid

PAGE ..... polyacrylamide gel electrophoresis

P. aeruginosa ..... *Pseudomonasa aeruginosa*

SDS ..... sodium dodecyl sulfate

ssDNA ..... single stranded deoxyribonucleic acid

S. aureus ..... *Staphylococcus aureus*

SELEX ..... Systematic evolution of ligands by exponential expansion

Tris ..... 2-amino-2-(hydroxy-methyl)-l,3-propanediol

V_{max} ..... maximal initial rate of a reaction

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To my angel, you know who you are

CHAPTER ONE

Introduction

Background

Penicillin

Penicillin was originally discovered by Sir Alexander Fleming in 1929 as an excretion from mold and was used clinically to treat bacterial infections from World War II onward. Penicillin is a β-lactam antibiotic that mimics the structure of D-Alanyl-D-alanine (Figure 1). β-lactam antibiotics bind to the active site of D-alanyl-D-alanine

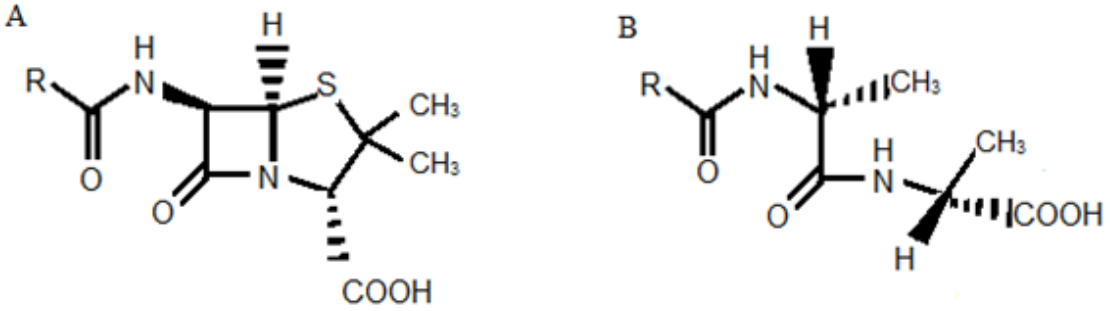

Figure 1. Comparison of (A) penicillin backbone and (B) D-Alanyl-D-alanine

transpeptidases (DD-peptidase) and inhibit the DD-peptidases' normal function of linking strands of peptidoglycan of bacterial cell wall together which contributes to the expansion of the cell wall (Waxman, et al., 1983).

β-Lactamase

β-lactamases are enzymes which are thought to have evolved from penicillin binding proteins such as DD-peptidase and show some sequence homology with the DD-peptidase family. *Bacillus cereus* (*B. cereus*) is a gram positive bacterium which

produces β-lactamases (β -lactamhydrolyases, EC 3.5.2.6) capable of deactivating β-lactam antimicrobial agents spanning from penicillin to third generation cephalosporins. β-lactamases deactivate penicillin and its derivatives by hydrolyzing the β-lactam ring within the antibiotic. The hydrolyzed compound can no longer be recognized by DD-peptidases, which will not incorporate the penicillin into the cell wall (Fisher, et al., 2005).

β-lactamases are split into classes A, B, C, and D. Classes A, C, and D contain a serine residue in the active site while class B contains either one or two zinc ions in the active site. Class A β-lactamases, such as β-lactamase I from *B. cereus* 569/H/9, tend to target penicillin using the serine residue in the active site as a nucleophile (Perez, et al., 2007; Walsh, et al., 2005).

β-lactamase genes can occur as a part of the chromosomal DNA (deoxyribonucleic acid) of a bacterium or as a portion of a plasmid carried by the bacterium. Chromosomal β-lactamase genes occur naturally in many genera of gram-negative bacteria and the first β-lactamase was identified in *Escherichia coli (E. coli)* (a gram-negative bacteria) before penicillin was applied in medical settings. Soon after the introduction of penicillin to the medical field, a resistant strain of *Staphylococcus aureus (S. aureus)* (a gram-positive bacteria) emerged due to a plasmid-encoded β-lactamase and was soon seen in most clinical isolates of *S. aureus*. Similarly, TEM-1 was the first plasmid-mediated β-lactamase in gram-negative bacteria to be described and was found in a clinical isolate strain of *E. coli*. Movement of TEM-1 enzyme genes are plasmid and transposon mediated and are transferred between *E. coli* and other species of *Enterobacteriaceae genera* such as *Enterobacter aerogenes, Morganella morganii,*

Proteus mirabilis, *Proteus rettgeri*, *Salmonella spp.*, as well as a few non-*Enterobacteriaceae* gram-negative bacteria such as *Pseudomonas aeruginosa (P. aeruginosa)* and *Capnocytophaga ochracea* (Jacobs, et al., 1986; Kuck, et al., 1989).

β-lactamase Inhibitors

While β-lactam antibiotics are some of the most commonly prescribed antibiotics to combat bacterial infection, the emergence of β-lactam resistant bacteria pose a serious threat to human health as they are rapidly becoming a national and international issue. Treatment of these infections is critical, and typically involves the use of a β-lactamase inhibitor alongside the β-lactam antibiotic. Current inhibitors of β-lactamases include clavulanic acid, sulbactam, and tazobactam (Figure 2) which bind to the active site of the

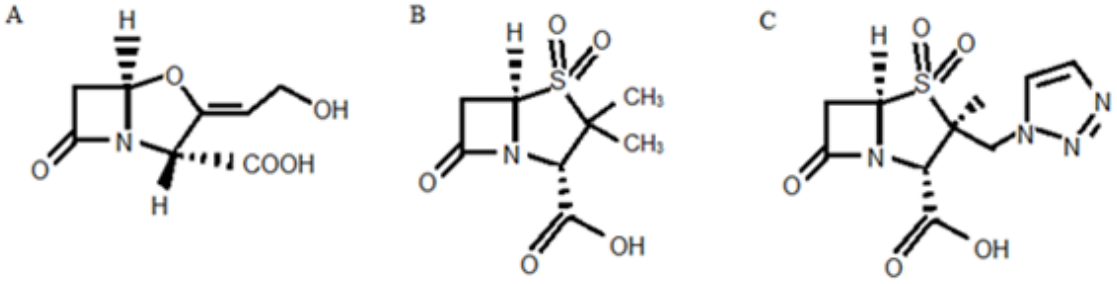

Figure 2 Structures of β-lactamase inhibitors: Clavulanic acid (A), Sulbactam (B), and Tazobactam (C)

β-lactamase and prevent the β-lactamase's ability to hydrolyze the β-lactam ring of the β-lactam antibiotic (Fisher, et al., 2005). When tested against members of the *Enterobacteriaceae* family, clavulanic acid and tazobactam show similar and efficient inhibitory ability with minimal inhibitory concentrations (MIC) in the low μM range, and both were superior to sulbactam in that they have a broader spectrum of activity when combined with the extended spectrum β-lactam piperacillin, with the exception of genera

Morganella where sulbactam and tazobactam were effective but clavulanic acid was not effective (Jacobs, et al. 1986; Kuck, et al. 1989).

There are numerous ways to describe the effectiveness of a compound as a drug, such as its MIC value, which is also involved in the calculation of therapeutic dose and therapeutic index. A therapeutic dose is determined by the MIC value and the ability of the therapeutic agent to penetrate tissues. The lower the MIC value, the lower the estimated required therapeutic dose. A drug's therapeutic index is determined by the lowest dose that is toxic to 50 percent of the patients (TD_{50}) and the minimum effective dose that is effective for 50 percent of the patients (MED_{50}) as shown in equation 1.

Therapeutic index= TD_{50}/ MED_{50} Equation 1.

The higher the therapeutic index the safer the chemotherapeutic agent. If the toxic dose remains the same and the therapeutic dose is decreased the therapeutic index increases (Willey, et al., 2010). For example, diazepam has a therapeutic index of greater than 100, but digoxin has a therapeutic index of 2 to 3. Diazepam is a much safer chemotherapeutic agent because it would take one hundred times more than the therapeutic dose to cause toxic side effects, while digoxin would only take twice the therapeutic dose to be toxic. Patients given therapeutic agents with extremely low therapeutic indexes must be monitored carefully for adverse reactions (Becker, 2007). A new inhibitor that requires a lower effective concentration and thus a lower therapeutic index would be more beneficial than the use of current β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Willey, et al., 2010).

Clavulanic acid. Though clavulanic acid is a good inhibitor of β-lactamases there are undesirable side effects that arise with the use of this drug. In a 2007 study by Salvo et. al, the adverse reactions for a combined therapy of amoxicillin (a penicillin) and clavulanic acid was compared to the adverse reactions of amoxicillin alone. The percentage of patients with an adverse reaction in the gastrointestinal system almost doubled in the clavulanic acid and amoxicillin group versus amoxicillin alone and quadrupled the percentage of patients with an adverse reaction in the hepatic system versus amoxicillin alone (Salvo, et. al, 2007). In a 1989 study by Kuck et. al, the MIC value that results in 90% inhibition (MIC_{90}) for piperacillin (an extended spectrum penicillin) administered with clavulanic acid against an oxacillin susceptible strain of *Staphylococcus aureus* (*S. aureus)* is 2.0 micrograms per milliliter (µg/mL) of piperacillin and 2.0 µg/mL of clavulanic acid, which results in a higher therapeutic index when compared to the MIC_{90} of piperacillin alone at 64 µg/mL (Kuck, et al., 1989).

Sulbactam. Combinational therapies with sulbactam and ampicillin work well for specific bacterial infections, such as pediatric bacterial meningitis, because of its ability to cross the blood brain barrier. However, sulbactam is not readily absorbed when administered orally, thus the most common form of administration is through injection. In the Kuck et al. study, the MIC_{90} for piperacillin and sulbactam against an oxacillin susceptible *S. aureus* was 4.0 µg/ml of piperacillin and 2.0 µg/ml of clavulanic acid.

Tazobactam. Tazobactam as an inhibitor in combination with piperacillin is the most recent of the three combinations to be approved. It is the most broad-spectrum combinational therapy and more effective than the clavulanic acid or sulbactam in

specific situations. Adverse effects with tazobactam and piperacillin are normally in the mild to moderate range and included stomach issues common to penicillin based antibiotics (Sanders, et al., 1996). In a 1989 study, the MIC_{90} for piperacillin and tazobactam against an oxacillin susceptible *S. aureus* is 2.0 µg/ml of piperacillin and 2.0 µg/ml of tazobactam.

Resistance to Current Inhibitors

Clinical isolates of *P. aeruginosa* have been identified as having chromosomal β-lactamases that are resistant to piperacillin-tazobactam treatments. Clinical isolates of *E. coli* have evolved to contain a TEM plasmid encoding a class A β-lactamases that are resistant to deactivation by clavulanic acid and sulbactam (Bradford, 2001; Harris, et al., 2002). There are numerous TEM plasmids that differ from TEM-1 by one or two point mutations in the amino acid sequence, and at least nineteen distinct inhibitor resistant TEM β-lactamases have been identified (Harris, et al., 2002).

The MIC for clavulanic acid, sulbactam, and tazobactam are in the µM range against the gram positive bacterium *S. aureus*, however a lower MIC and therapeutic index in a novel inhibitor could possibly result in fewer side effects. Therefore, with the combination of emerging resistance to current inhibitors and the toxic side effects of the current inhibitors, it is necessary to find new inhibitors of β-lactamases to ensure that β-lactam antibiotics continue to be effective against infectious strains (Harris, et al., 2002).

Reversible Inhibition

In Voet et al (2008), an inhibitor is defined as "substances that reduce an enzyme's activity… by combining with it in a way that influences the binding of

substrate and or its turnover number." An inhibitor that binds and subsequently releases from the enzyme is a reversible inhibitor. An inhibitor reversibly interacts with the enzyme by binding to the active site due to substrate mimicking, by binding with the enzyme in an area that is not the active site and causes a reversible conformational change in the enzyme, or by binding to the substrate:enzyme complex. These mechanisms of action are referred to as competitive inhibition, noncompetitive inhibition, and uncompetitive inhibition respectively. The type of reversible inhibition is determined by where the inhibitor binds to the enzyme and when the binding occurs (Voet, et al., 2008).

Competitive inhibition. Competitive inhibition occurs when an inhibitor competes with the substrate for the active site of the enzyme (Figure 3). A competitive inhibitor

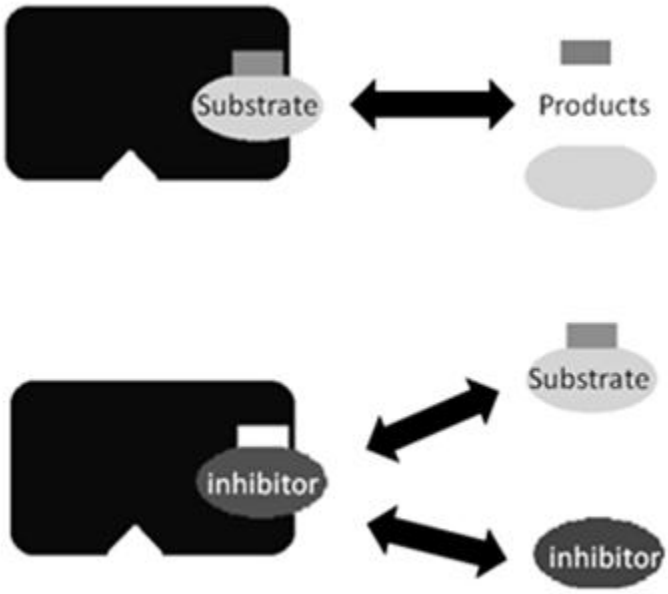

Figure 3. Competitive inhibition

often mimics portions of the substrate so that the enzyme will specifically bind to the

inhibitor, but fails to react in the same way as the substrate. Product inhibition is commonly utilized by cells to regulate enzyme functions because the product competes with the substrate for the active site of the enzyme and prevents over production. Transitional state analog competitive inhibition occurs when a compound mimics the structure of the substrate in the enzyme's transition state, which normally occurs during the substrate to product conversion (Voet, et al., 2008). An example of the graphical representation of competitive inhibition can be seen in Figure 4.

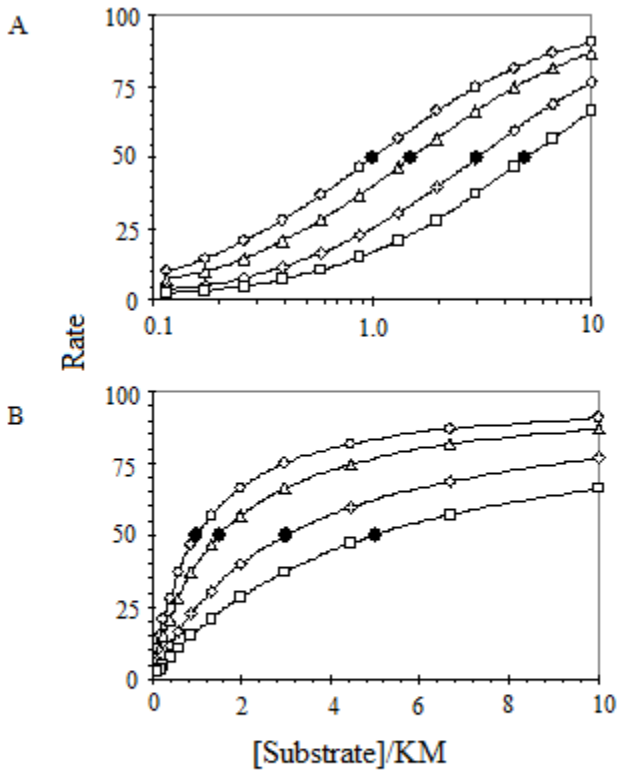

Figure 4. Competitive Inhibition, ○: no inhibitor added; Δ: 0.5xK_i inhibitor added; ◊: 2.0xK_i inhibitor added; □: 4.0xK_i inhibitor added; ●: Shift in apparent K_m for each binding modality (A) Logarithmic analysis, (B) Linear analysis (Adapted from Assay Guidance Manual Version 5.0, 2008)

Noncompetitive inhibition. In comparison, noncompetitive inhibition occurs when the inhibitor binds to an area of the enzyme that is not a part of the active site (Figure 5). The inhibitor binding changes the three-dimensional structure of the enzyme

and prevents the substrate from binding to the active site of the enzyme (Voet, et al., 2008). An example of the graphical representation of noncompetitive inhibition can be seen in Figure 6.

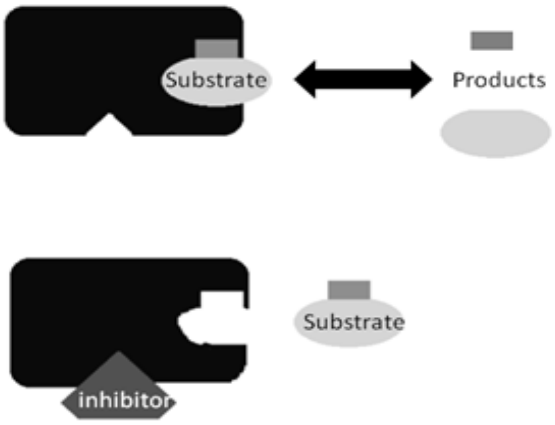

Figure 5. Noncompetitive Inhibition

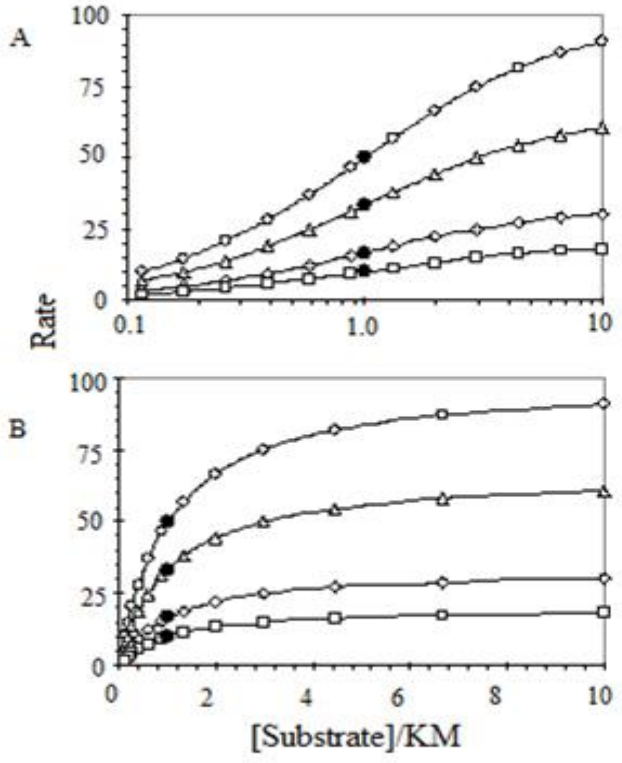

Figure 6. Noncompetitive Inhibition, ○: no inhibitor added; Δ: 0.5 x K_i inhibitor added; ◊: 2.0 x K_i inhibitor added; □: 4.0 x K_i inhibitor added; ●: Shift in apparent K_m for each binding modality (A) Logarithmic analysis, (B) Linear analysis (Adapted from Assay Guidance Manual Version 5.0, 2008)

Uncompetitive inhibition. Uncompetitive inhibition occurs when an inhibitor binds to the substrate:enzyme complex but not to the enzyme alone (Figure 7). The inhibitor binds to the enzyme substrate complex and creates a structural change in the enzyme which prevents the action of the enzyme (Voet, et al., 2008). An example of the graphical representation of uncompetitive inhibition can be seen in Figure 8.

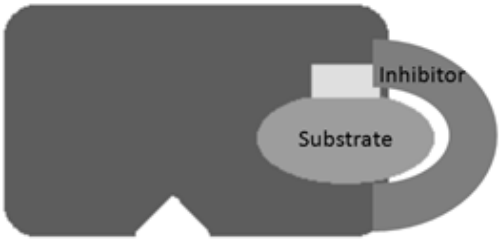

Figure 7. Uncompetitive inhibition

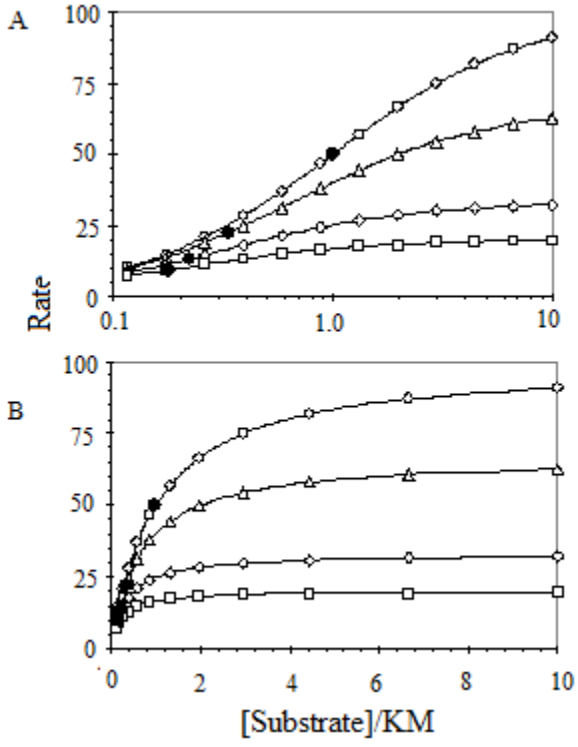

Figure 8. Uncompetitive Inhibition, ○: no inhibitor added; Δ: $0.5xK_i$ inhibitor added; ◊: $2.0xK_i$ inhibitor added; □: $4.0xK_i$ inhibitor added; ●: Shift in apparent K_m for each binding modality (A) Logarithmic analysis, (B) Linear analysis (Adapted from Assay Guidance Manual Version 5.0, 2008)

Irreversible Inhibition

Irreversible inhibition occurs when an inhibitor binds so tightly to an enzyme or covalently binds to an enzyme that the function of the enzyme is permanently blocked. Examples of irreversible inhibitors are compounds that are used to identify serine and histidine residues of serine proteases (Voet, et al., 2008).

SELEX

Systematic evolution of ligands by exponential enrichment (SELEX) is performed in cycles known as rounds. Each SELEX round consists of the amplification of DNA or RNA oligonucleotides, incubation with the target, selection of oligonucleotides that bind with high affinity, and isolation of the higher affinity oligonucleotides (Figure 9). In the first round, a pool of randomly generated oligonucleotides is incubated with the target

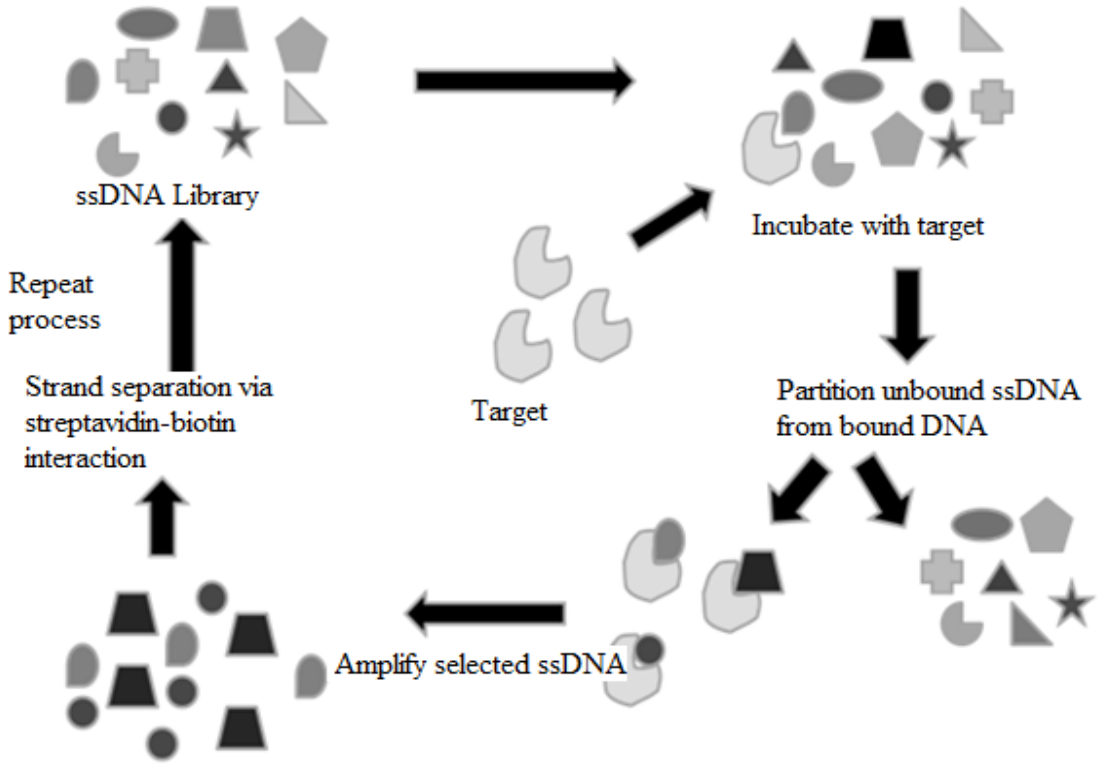

Figure 9: SELEX process

enzyme, but in subsequent rounds the oligonucleotide pool is formed by PCR of the tighter binding oligonucleotides selected by the previous round.

In single stranded deoxyribonucleic acid (ssDNA) SELEX, the amplification of the isolated DNA requires an extra step to regenerate the oligonucleotides in ssDNA form instead of double stranded deoxyribonucleic acid (dsDNA). After the chosen number of rounds are completed, the isolated, tight binding ssDNAs are referred to as aptamers and are subsequently cloned and sequenced (Stoltenburg, et al., 2007).

ssDNA Secondary Structures

Though dsDNA always forms a helical structure, ssDNA is able to form secondary structures depending on their nucleotide sequence and the spontaneity properties of the secondary structure. Spontaneity properties can be determined by the change in Gibbs free energy equation (Equation 2).

$$\Delta G = \Delta H - T\Delta S \qquad \text{Equation 2}$$

ΔG is the change in free energy, ΔH is change in enthalpy (heat), T is temperature, and ΔS is the change in entropy (change in randomness of a system). If ΔG is negative the systems change (eg structure folding) is spontaneous because no energy is required to create that change, however if ΔG is positive energy is nonspontaneous because energy is required to cause the system change (Voet, et al., 2008). An example of spontaneous changes in ssDNA structures is the formation of hairpin loops. The secondary structures of ssDNA and the energy required to create the secondary structure can be determined by using the program Mfold (Zucker, 2003).

The secondary structures created can then be examined to determine potentially important regions of the ssDNA. The original sequence can be split into smaller sections

containing the potential important regions. These smaller sequences can then be evaluated for inhibition and determine the important portions of the sequence for inhibition.

Rationale and Significance

To manage bacterial resistance to current antibiotics, new antibiotics must be used. The ability of β-lactam antibiotics to interrupt cell wall formation is well known and very effective when β-lactamases are not present. Through the utilization of inhibitors of β-lactamases, β-lactams can once again be used as antibiotic agents (Fisher, et al., 2005). Since current inhibitors produce undesirable side effects and are difficult to administer, new inhibitors are necessary to treat infections by these β-lactam and inhibitor resistant strains.

The SELEX process can produce aptamers that strongly bind to the enzyme and cause conformational change to create new inhibitors. The difference between the current inhibitors and the SELEX created oligonucleotide inhibitor would enable the oligonucleotide to avoid the bacterial resistance genes that target the structures of the current inhibitors.

In this project SELEX was used to create aptamers that bind to the enzyme β-lactamases I from *B. cereus* 569/H/9 and cause inhibition, and thus new inhibitors of β-lactamases were created. The aptamers were studied to determine the inhibition pattern and structure.

CHAPTER TWO

Methods

DNA

Labeling of DNA Strands

Polymerase Chain Reaction (PCR) was used to create dsDNA molecules with only the forward primer being biotinylated. For the first round of SELEX, an original DNA pool was used; the DNA in this pool is characterized by a 30 base pair random sequence flanked by two known 15 base pair sequences which include primer binding sites as well as known restriction enzyme sites.

The DNA was amplified using PCR. 10 µL of Go-Taq Master Mix® (Promega©), 150 pmol each of biotinylated forward primer, non-biotinylated reverse primer, and DNA template, and 7 µL of nuclease free water were combined in a eppendorf tube and placed into the Thermocycler (Eppendorf©). The PCR technique includes: 94°C for 3 minutes, then cycle for 20 times (94°C for 40 sec, 56°C for 40 sec, 72°C for 20 sec), 72°C for 5 min, and finally a 4°C hold. The subsequent rounds of SELEX amplification replace the 1 µL of DNA template and 7 µL of nuclease free water with 8 µL of the selected ssDNA from the separation stage. After each amplification step a subsequent ssDNA formation step was completed.

Separation of dsDNA into Isolated ssDNA

ssDNA was formed by utilizing the strong binding of streptavidin and biotin. Paramagnetic beads were employed (New England Biolab Inc.), in which the surface of a Fe^{+} bead has been coated with streptavidin. The biotin on the forward DNA strands binds to the streptavidin molecule during a 30 minute incubation of the beads and PCR product. The optimum temperature for binding was found to be 22°C; if the beads are incubated at a temperature of 37°C or 4°C no significant binding occurs and very little ssDNA is recovered.

After incubation, the beads along with the newly bound DNA strands are separated from the supernatant of the PCR mix by using a magnet to cause the Fe^{+} beads to form a pellet. The non-biotinylated reverse strands of DNA were unbound from the biotinylated forward strands by 90 sec incubation with 0.1 M NaOH, which causes the hydrogen bonds between the DNA strands to weaken and release, allowing the formation of a single stranded DNA molecule. However, the biotin-avidin bond remains unaffected and the newly formed biotinylated ssDNA remains bound to the Fe^{+} bead, thus enabling isolation.

The supernatant was removed and the pellet was resuspended and washed with autoclaved distilled water. Again the magnet was used to form a Fe^{+} bead pellet and the supernatant was removed. The remaining pellet was resuspended in autoclaved distilled water and the tube with the solution was suspended in a 70°C water bath for 90 seconds to break the streptavidin-biotin interaction. The Fe^{+} beads were pelleted by applying the magnet and the supernatant was placed into a fresh tube. The resulting pellet was retreated with heat to remove any bond ssDNA (Espelund, et al., 1990). The supernatant

containing the newly formed ssDNA was quantified by ultraviolet-visible (UV-vis) spectroscopy and by 6% native polyacrylamide gel electrophoresis (PAGE) which was stained with ethidium bromide to detect both the size and the amount of the ssDNA.

Preparation of Protein

β-lactamase I from *B. cereus* 569/H/9 was previously purified and provided by Texas Tech University. The concentration of the protein was determined with bovine serum albumin (BSA) as the standard in conjunction with the Bradford method (Bradford, 1976). Purity was check by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), which results in one single band at molecular weight of approximately 28,000 Da. The protein solution underwent the Bradford method and the concentration was found to be 2.9 µg/mL. Protein solutions were aliquotted into eppendorf tubes and frozen at -80°C for future use.

Separation of the Complex (β-lactamase I:ssDNA) from Unbound ssDNA

The absorbance of the ssDNA was measured at 260nm to a concentration of 7 µg/mL in the initial round. All subsequent rounds were diluted or concentrated to 7 µg/mL. In an eppendorf tube, 40 µL of ssDNA solution, 5.5 µL of 100 mM 2-amino-2-(hydroxy-methyl)-l,3-propanediol (Tris), and 10 µL of protein solution were combined and incubated for 30 minutes at room temperature. After incubation, the solution was combined with 2 mL of 10 mM Tris and added to a centrifuge filter tube with a 30,000 dalton pore size. The 30,000 dalton pore size allows the protein and the ssDNA bound to the protein to remain above the filter while the unbound DNA passes through the membrane.

The filter tube was placed in the centrifuge for 20 minutes or until about 55.5 µL of protein solution is left. 8 µL of the protein and bound ssDNA solution was then added to 10 µL of Go-Taq Master Mix®, 1 µL of reverse primer and 1 µL of Biotinylated forward primer to be used in the amplification step of SELEX. To enhance the specificity of the aptamers, increasing concentrations of NaCl was added (Stoltenburg, et al., 2007).

Sequencing of Aptamers

The ssDNA that was selected for during the SELEX rounds was amplified via PCR. The PCR solution consisted of 10µL of Go-Taq Master Mix©, 1µL of reverse primer, 1µL of forward primer, 2 µL of SELEX ssDNA, and 6µL of autoclaved distilled water. The Thermal Cycler ran the previously explained SELEX cycle for 40 rounds. The resulting PCR product was run on a 6% native PAGE . The native PAGE was stained with ethidium bromide and placed on a UV-lamp where the DNA band was excised. Crush and Soak was performed on the DNA band to remove it from the native PAGE-gel (Sambrook, et al., 1989). After crush and soak, the DNA was purified from the salt solution via ethanol precipitation and resuspended in autoclaved distilled water. A restriction digest of the DNA and subsequent cloning into the plasmid pET28b plasmid was performed using BamHI and HindIII as restriction enzymes.

Ligation of the pET28b plasmid with the DNA was completed using T4 ligase. The newly ligated plasmid was cloned into *E. coli* DH5α competent cells using heat shock. The cloned cells were plated onto a kanamycin antibiotic plate to select for bacteria that contains the pET28b plasmid. The colonies that form on the plate were individually selected and each used to inoculate 10 mL of Luria-Bertani (LB) media

containing some concentration of kanamycin. After overnight incubation, the resulting cultures were spun down and the supernatant discarded. Some of the cells were used for glycerol stock for later use; the rest of the cell underwent plasmid extraction using the Qiagen miniprep plasmid extraction kit. Sequencing of plasmids took place at Integrated DNA Technologies and the resulting sequence was analyzed to digitally remove the inserted aptamer sequences from the plasmid sequence.

Sequences

The aptamer sequences for two aptamers, a 22-mer and a 28-mer, were analyzed using a sequence alignment program (e.g., BioEdit) and using a secondary structure program (e.g., MFold) to predict the secondary structure of the aptamer. Secondary structures give the aptamers more variability and can cause the aptamer to bind more tightly to the target.

Enzyme Activity Assay

Enzyme assays were performed in 1 cm pathlength quartz cuvettes, and reaction mixtures contained 500 μL 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.0), 2 μL of 2.9 mg/mL Bla1, varying volumes of 50 mM Penicillin G (in water), and amount of water necessary to bring the total volume to 1 mL (final MOPS concentration of 50 mM). The assay was performed at 25 °C and reactions were initiated by the addition of enzyme. One activity unit is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μM of Penicillin G in one minute at 25 °C. Reactions were monitored by measuring the decrease in absorbance at 232 nm by UV-vis spectrophotometry (UV-2450, Shimdzu) due to the hydrolysis of Penicillin G, the molar

extinction coefficient of which is 1100 $M^{-1}cm^{-1}$ at 232 nm (Bouthors, et al., 1999). Initial velocity rates were calculated based on this molar extinction coefficient. Several assays were performed, with the varying penicillin G concentration, to determine the average change in absorbance per second. The data was collected by the UV Probe 2.21 program. The data was then analyzed by Enzyme Kinetics (Sigma Plot 2001).

Inhibition of β-lactamase I by 22-mer ssDNA

Numerous assays were performed and repeated to determine the inhibitory effect of the various found aptamers. Kinetic constants that were determined are the dissociation constant for inhibitor from enzyme-inhibitor complex (K_i), the Michaelis-Menten constant (K_m), maximal initial rate of a reaction (V_{max}), and inhibitor concentration necessary to produce 50% inhibition of enzyme activity(IC_{50}) of β-lactamase I's by the SELEX 22-mer.

Mfold Analysis

The sequences of the 22-mer and 28-mer were analyzed using the Mfold function of OligoAnalyzer 3.1 from Integrated DNA Technologies to predict secondary structures and the spontaneity of the secondary structures of the 22-mer and the 28-mer (Zuker, 2003). The secondary structures of each were analyzed and the 22-mer was broken down into a 10-mer and an 11-mer component. The 10-mer and 11-mer components were analyzed by using Mfold and their secondary structures and the spontaneity of the secondary structures were predicted.

Inhibition of β-lactamase I by 10-mer and 11-mer ssDNA

Numerous assays were performed and repeated to determine the inhibition values. Kinetic constants that were determined were the K_i, the K_m, and V_{max}, of β-lactamase's inhibition by the 10-mer and 11-mer.

CHAPTER THREE

Results

ssDNA Formation

A random ssDNA oligonucleotide pool was synthesized by Integrated DNA Technologies, with a total oligonucleotide length of 60 base pairs. The complimentary strands of the 60-mer were synthesized by PCR using biotinylated forward primers to amplify the DNA and produce double stranded DNA. The PCR products were then isolated using streptavidin beads and underwent isolation of the biotinylated ssDNA forward strands from the ssDNA reverse strands. The ssDNA forward bands were purified using native PAGE-gel. Figure 10A shows the dsDNA oligomers in comparison

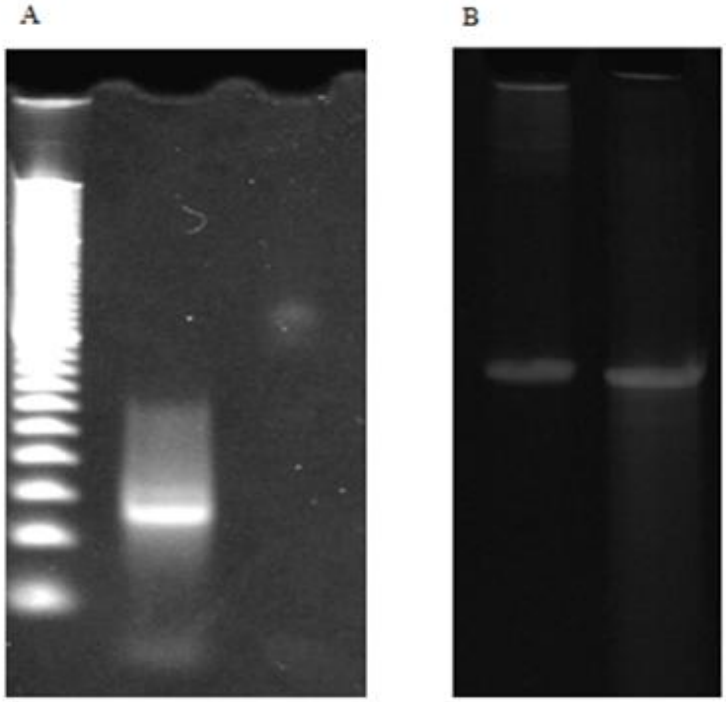

Figure 10. (A) Comparison of dsDNA and ssDNA in a Native PAGE-gel, Lane 1: 20 base pair ladder, Lane 2: dsDNA 60-mer, Lane 3: ssDNA 60-mer, (B) ssDNA and dsDNA in Urea gel; Lane 1: ssDNA, Lane 2: dsDNA

to the ssDNA oligomers that were produced and Figure 10B shows ssDNA and dsDNA in denaturing urea-polyacrylamide gel.

SELEX Method

SELEX method was used to select high affinity aptamers. β-lactamase I was incubated with the 60-mers for 30 minutes and then unbound ssDNA was removed by using filter concentration method. The resulting bound ssDNA:β-lactamase I then underwent amplification using PCR. The PCR product was then subjected to ssDNA formation to begin the next round of SELEX.

Ten rounds of SELEX were performed using increasing concentrations of NaCl to select for aptamers of tighter binding affinity during the course of the rounds (Table 1).

Table 1. Concentration of NaCl in solution during SELEX binding.

Round	[NaCl]
1	0 mM
2	0 mM
3	10 mM
4	10 mM
5	20 mM
6	20 mM
7	40 mM
8	40 mM
9	80 mM
10	80 mM

After completing 10 rounds of SELEX the ssDNA aptamers were again incubated with the β-lactamase I for 30 minutes and then run on a native PAGE-gel. The complex of ssDNA:β-lactamase I can be seen as a complex in the upper part of the native PAGE-gel (Figure 11).

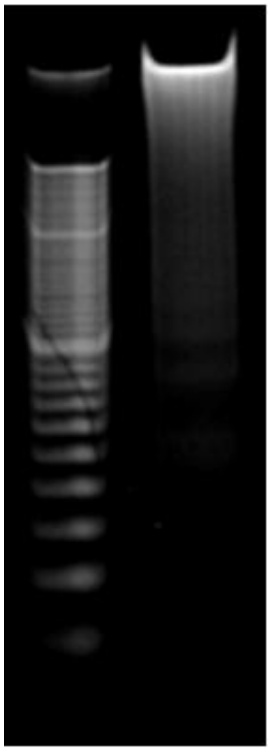

Figure 11. ssDNA:β-lactamase I complex in 6% native PAGE gel; Lane 1: 20 base pair DNA ladder, Lane 2:ssDNA:β-lactamase I complex

Sequences

The ten round SELEX process led to four ssDNA sequences which included two 28-mers, one 9-mer, and one 22-mer. These ssDNA lengths differ from the original 30 random base DNA sequence due to infidelity of the DNA polymerase which results in 1 base substitution errors per 9,000 bases and 1 frameshift error (insertion or deletion) per 41,000 bases. Since the sequences went through numerous PCR amplification rounds during the SELEX procedure some errors should be expected (Tindall, et al., 1988). The

two 28-mers were almost identical with only one guanine to cytosine substitution at base 26. Both guanine and cytosine have the same number (three) of structural areas that are able to form hydrogen bonds, thus the two sequences were treated as one sequence. Out of the two 28-mers it was decided that sequence 28-mer A would be used for inhibition studies. The 9-mer was not evaluated further because of its severely truncated form. The 22-mer was also selected for inhibition studies. The four sequences that resulted from the SELEX treatment can be seen in Table 2. The 22-mer and 28-merA sequences were synthesized by Integrated DNA Technologies to be used for further analysis.

Table 2: SELEX sequences

Name	Sequence
22-mer	5'TGGCCATCGCCTATCTTCCCCC3'
28-mer A	5'GACGAAGGGAGACGATACACCCAATGTT3'
28-mer B	5'GACGAAGGGAGACGATACACCCAATCTT3'
9-mer	5'ACACCACAG3'

Mfold Analysis

The sequences 22-mer and 28-mer were analyzed using Mfold (OligoAnalyzer 3.1 from Integrated DNA Technologies) to predict spontaneous secondary structures of 22-mer and 28-mer (Table 3). One secondary structure of the 22-mer was predicted and

Table 3. Spontaneity of Mfold predicted secondary structures of individual sequences (Zuker, 2003)

Sequence Name	ΔG (Kj/mol)
22-mer	-2.27
28-mer structure 1	-1.95
28-mer structure 2	-1.95

contained a 10-mer stem-loop region, an unfolded 11-mer region, and a ΔG of -2.27 kilojoules/mole (Kj/mol) (Figure 12). The secondary structure of the 10-mer was

Figure 12. Predicted structure of 22-mer calculated by MFold program (Zuker 2003)

predicted by MFold program and confirmed the stem-loop structure seen in the 22-mer predicted structure, but was unable to predict a secondary structure for the 11-mer. The predictions for the 28-mer by Mfold were that of two different secondary structures which both had ΔG of -1.95(Kj/mol) (Figure 13).

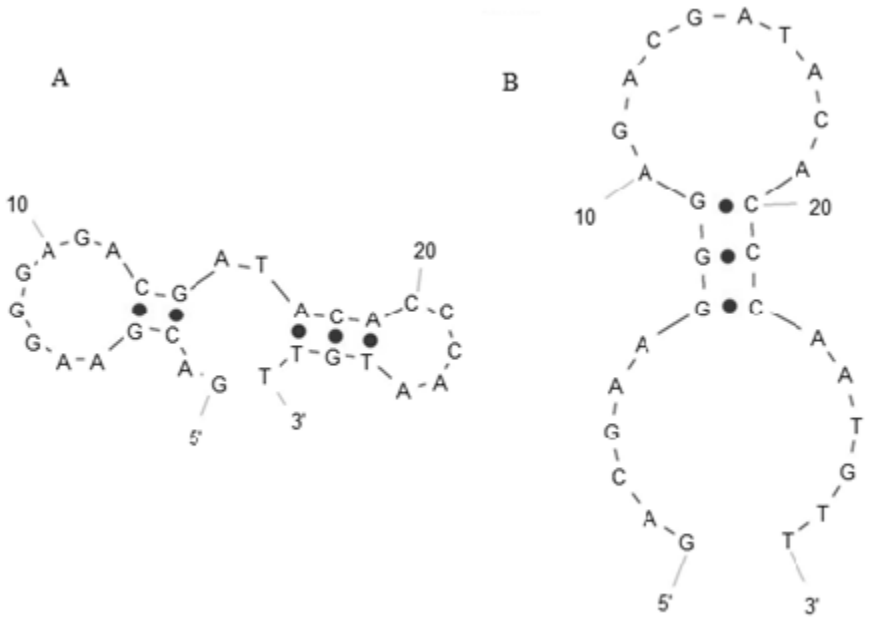

Figure 13. Predicted structures of 28-mer calculated by MFold program (Zuker 2003)

Enzyme Activity Assay

Enzyme assay was performed to determine K_m and V_{max} values (see Chapter 2). The data points are fitted to Michaelis-Menten equation (Equation 3) using the Enzyme Kinetics program in Sigma Plot 2001.

$$v_o = (V_{max} \times [S])/(K_M + [S]) \qquad \text{Equation 3.}$$

Where v_o is initial velocity and [S] is substrate concentration.

By this analysis, the β-lactamase I from *B. cereus* has a K_m of 333 ± 53.3 mM and a V_{max} of 20.1 μmol/min/mg with penicillin G as a substrate (Figure 14).

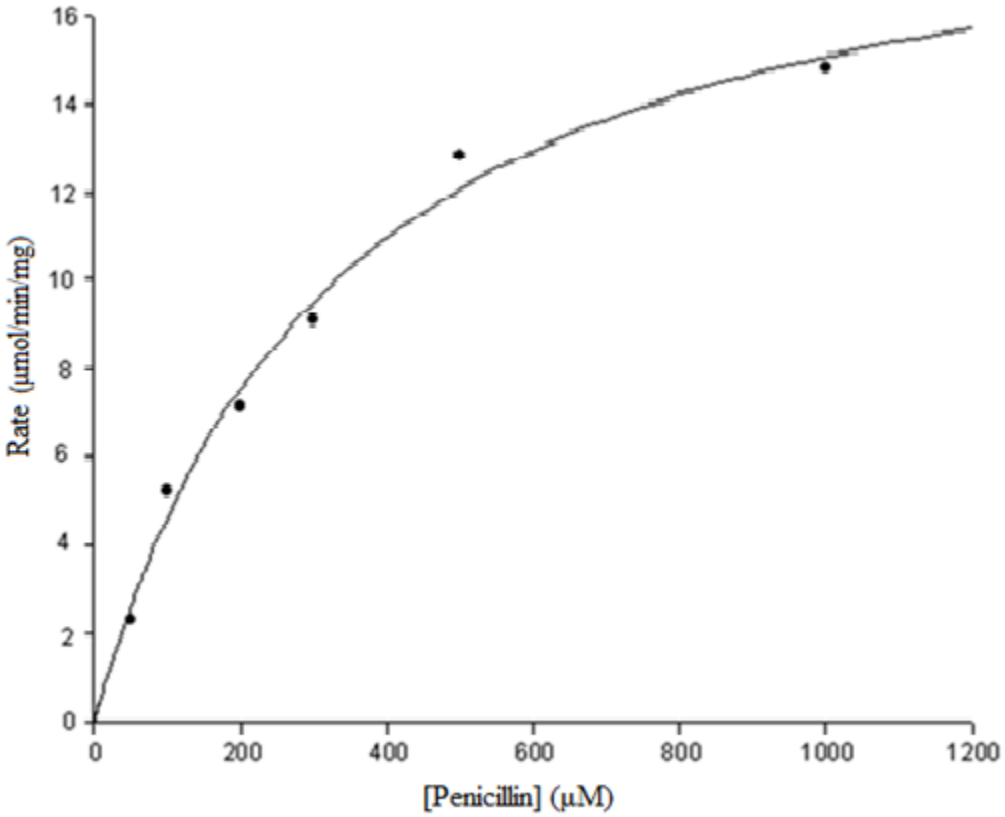

Figure 14. Rate of Penicillin breakdown over various concentrations of Penicillin G.

22-mer and 28-mer Inhibition Assays

22-mer showed inhibition with a K_i of 15.6 nM, K_m of 213.5 mM, and a V_{max} of 9.8 µmol/min/mg (Figure 15). Due to the inhibition shown by the 22-mer sequence, 22-mer inhibition was evaluated further and the 10-mer and 11-mer components were synthesized by Integrated DNA Technologies.

The 28-mer over increasing concentrations of aptamer and penicillin G at 1000 µM concentration failed to show consistent inhibition by β-lactamase I (Figure 16). Due to the lack of inhibition of β-lactamase I by 28-mer, the inhibition of β-lactamase I by sequence 28-mer was not evaluated further.

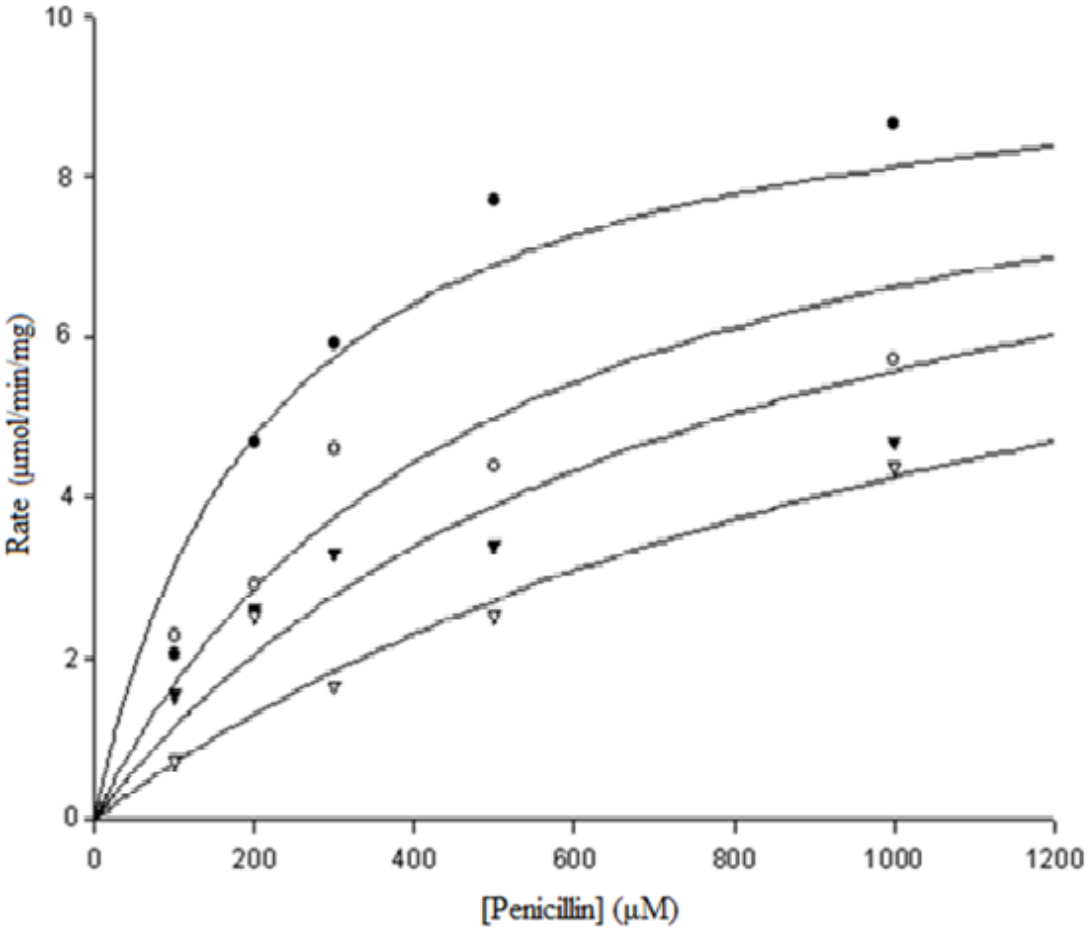

Figure 15. Rate of Penicillin breakdown compared with the concentrations of 22-mer of ● 0 nM, o 20 nM, ▼ 40 nM, and ▽80 nM and varying concentrations of Penicillin.

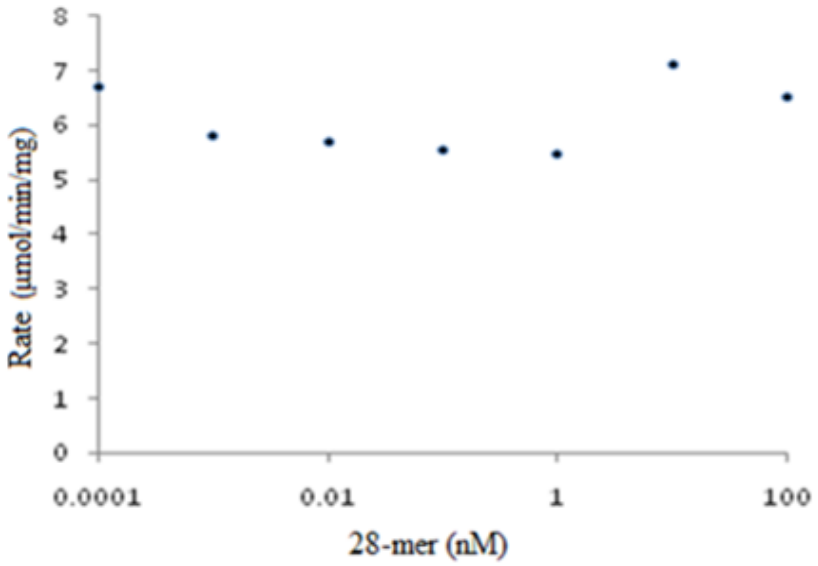

Figure 16. Log scale depicting the rate of penicillin G breakdown compared with varying concentrations of 28-mer.

Cephalexin Inhibition Pattern

Cephalexin showed a competitive inhibition pattern against β-lactamase I during a steady-state kinetic study (Figure 17). The value of K_i for Cephalexin was 58.9 mM. In a comparable study conducted in 1956, Cephalexin had a K_i of less than 0.3 mM, however the hydrolysis of penicillin was measured indirectly through CO_2 production instead of the absorption of light at 260 nm and the enzyme was in solution containing aqueous gelatin instead of MOPS buffer (Abraham, et al., 1956).

22-mer Inhibition Pattern

The 22-mer also showed a competitive inhibition pattern of β-lactamase I (Figure 18). The K_i value for 22-mer was 15.6 nM.

Mfold Analysis of 22-mer Components

Spontaneity properties of 10-mer and 11-mer can be found in Table 4 and the Mfold secondary structure of 10-mer can be found in Figure 19.

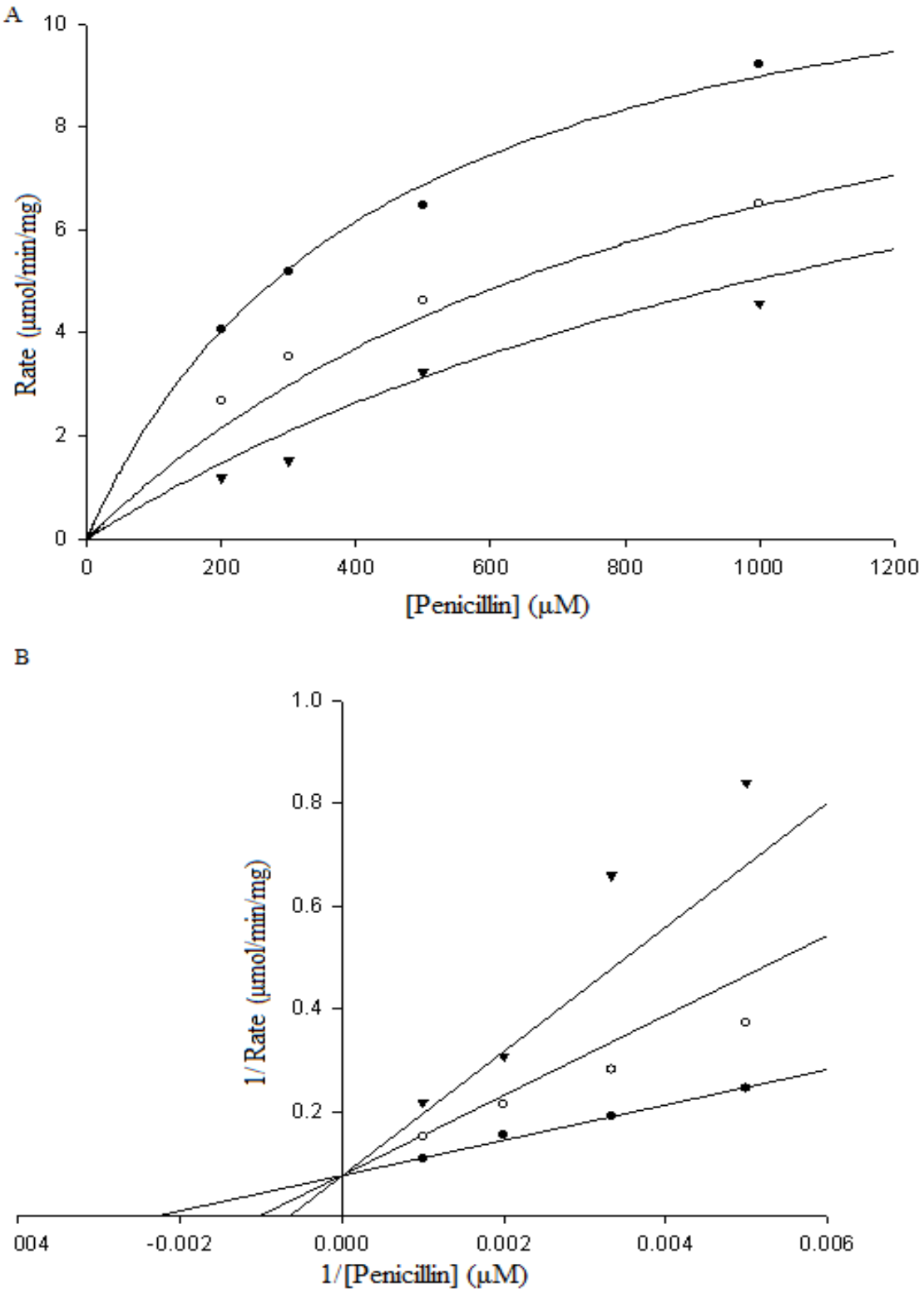

Figure 17. Michaelis Menten (A), Lineweaver-Burk (B) of the inhibition of β-lactamase I by Cephalexin: ● 0 mM Cephalexin, o 75 mM Cephalexin, ▼ 150 mM Cephalexin

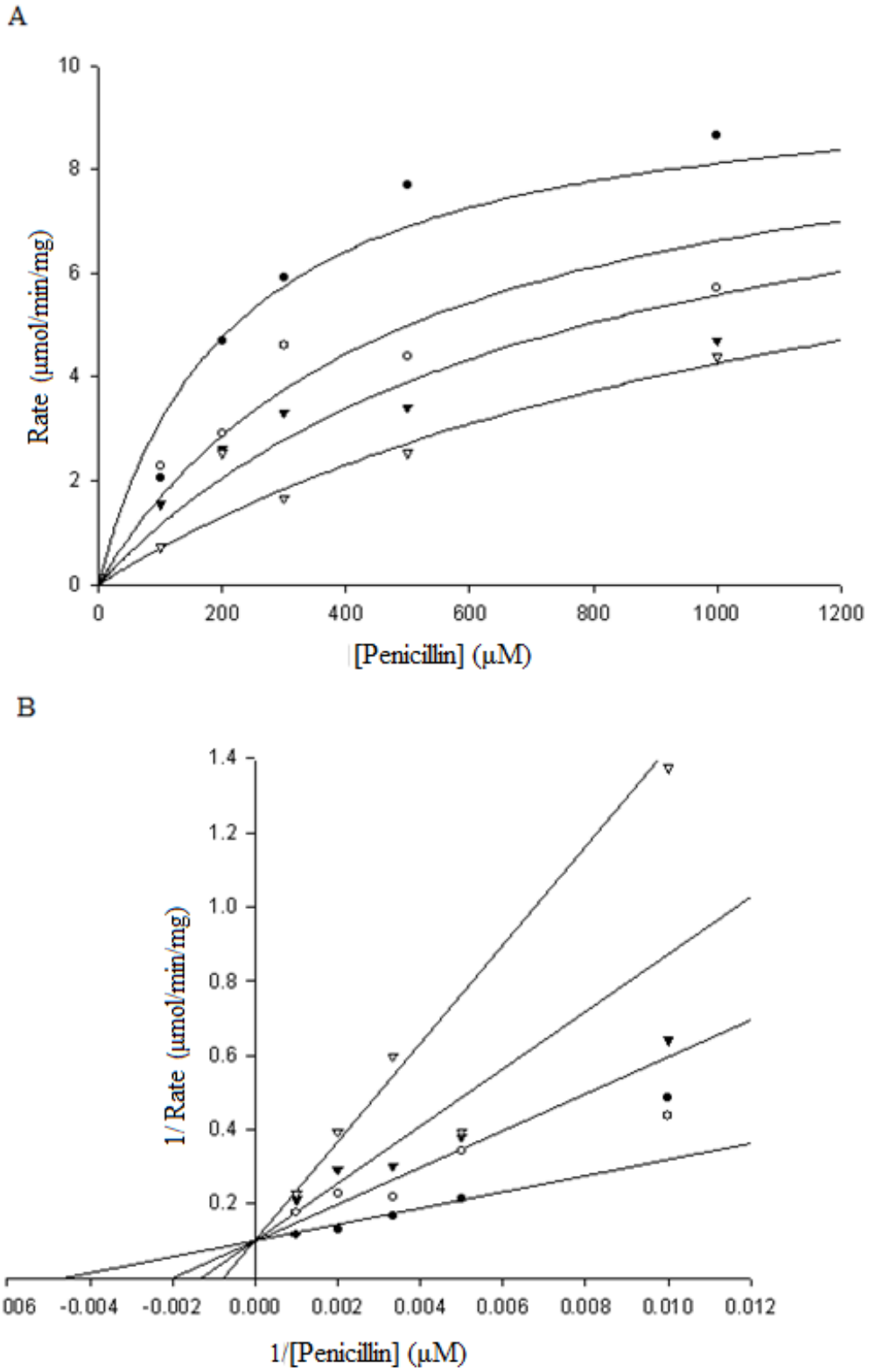

Figure 18. (A) Michaelis-Menten plot of the inhibition of β-lactamase I by 22-mer (B) Lineweaver-Burk plot of the inhibition of β-lactamase I by 22-mer: ● 0 nM of 22-mer, o 20 nM of 22-mer, ▼ 40 nM of 22-mer, ▽ 80 nM of 22-mer.

Table 4. Spontaneity of 10-mer and 11-mer sequences

Name	ΔG (Kj/mol)	Sequence
10-mer	-1.12	5'GGCCATCGCC3'
11-mer	0	5'TATCTTCCCCC3'

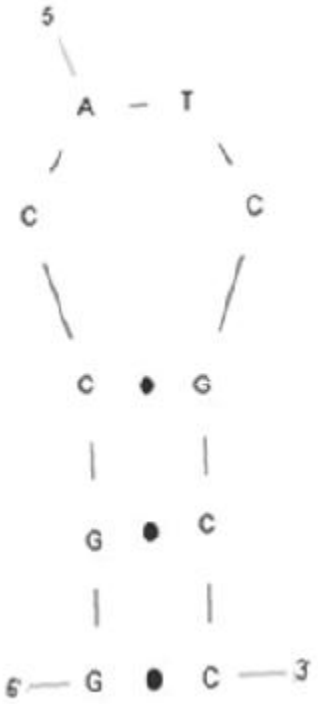

Figure 19. Predicted structure of 10-mer calculated by MFold program (Zuker 2003)

10-mer and 11-mer Inhibition Assays

The sequences of 10-mer and 11-mer were synthesized by Integrated DNA Technologies. 10-mer showed a competitive inhibition pattern of β-lactamase I during a steady-state kinetic study (Figure 20). The K_i value for the 10-mer was 261.7 nM.

In comparison to the other potential inhibitors, the 11-mer showed no significant inhibition of β-lactamase I during its steady-state kinetic study (Figure 21).

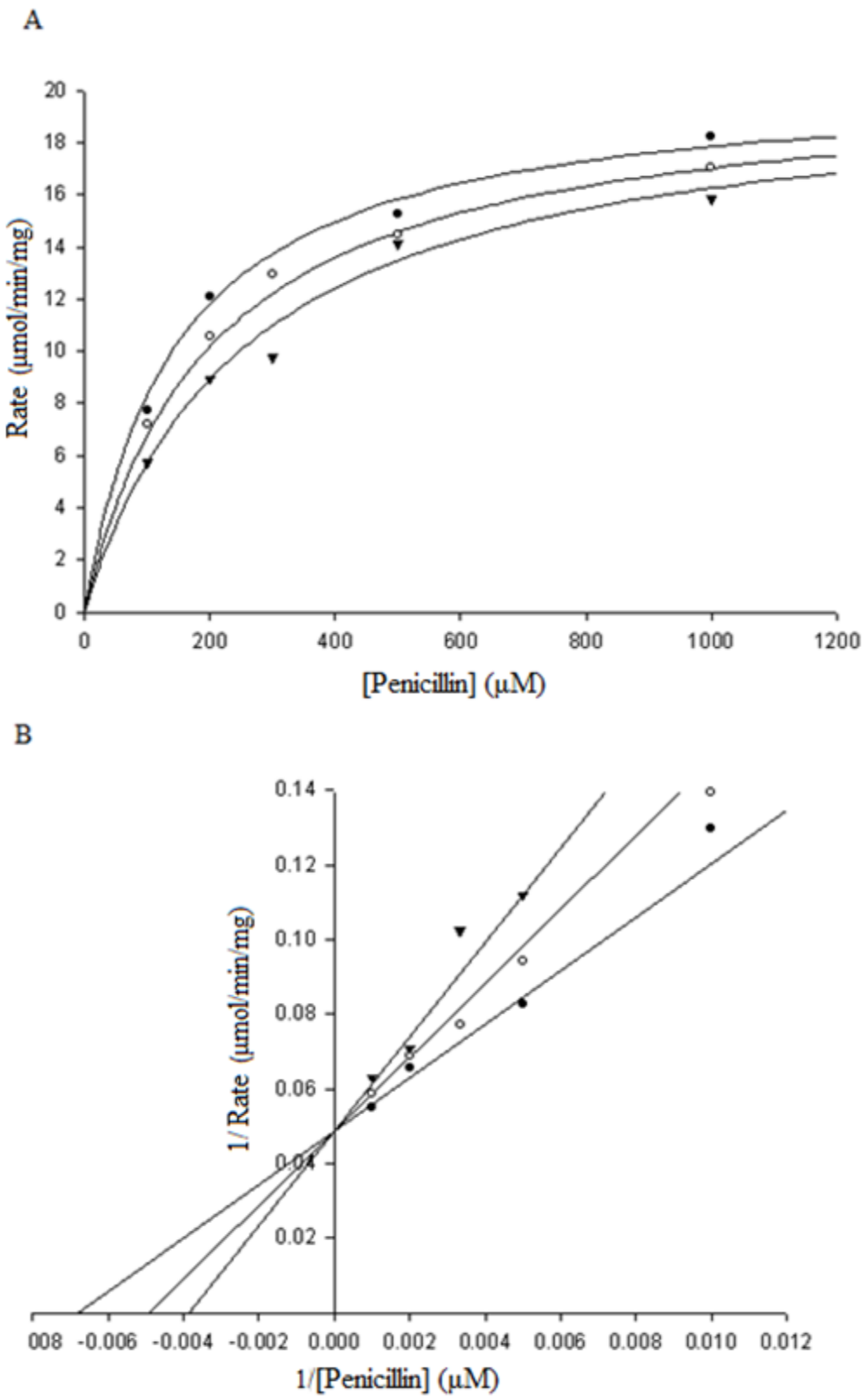

Figure 20. (A) Michaelis-Menten plot of the inhibition of β-lactamase I by 10-mer (B) Lineweaver-Burk plot of the inhibition of β-lactamase I by 10-mer: ● 0 nM of 10-mer, o 100 nM of 10-mer, ▼ 200 nM of 10-mer

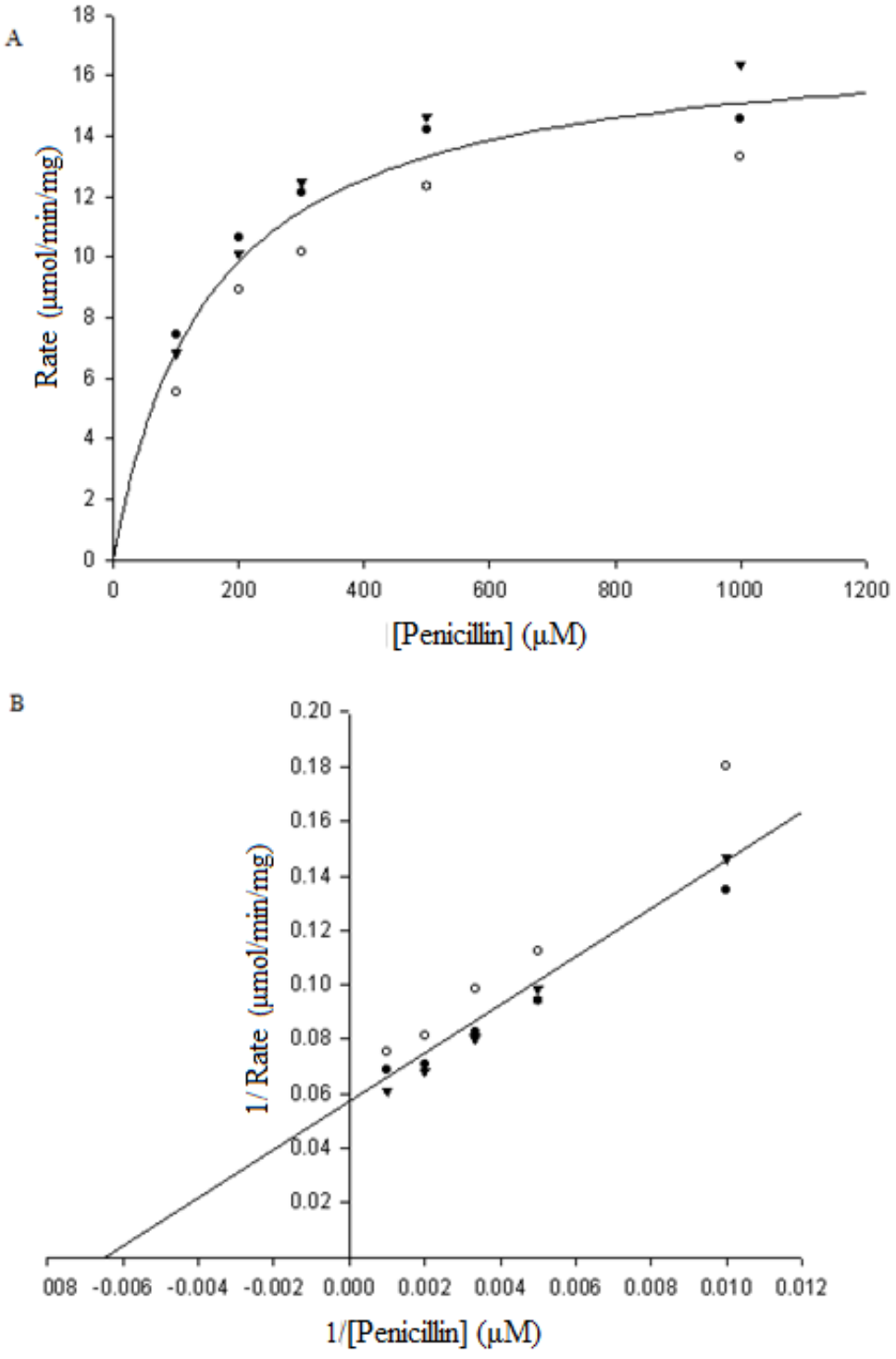

Figure 21. (A) Michaelis-Menten plot of the inhibition of β-lactamase I by 11-mer (B) Lineweaver-Burk plot of the inhibition of β-lactamase I by 11-mer: ● 0 nM of 11-mer, o 100 nM of 11-mer, ▼ 200 nM of 11-mer

CHAPTER FOUR

Discussion

SELEX procedures enabled the isolation of high-affinity oligonucleotides that can inhibit a class A β-lactamase. By increasing the salt concentrations during the SELEX rounds an increase in stringency of selection was created, and non-specific-binding oligonucleotides were eliminated leaving two ssDNA molecules after ten rounds of SELEX. A comparison of the two ssDNA molecules' K_i values enabled the elimination of one of the ssDNA and in reviewing of the inhibition pattern of the remaining 22-mer ssDNA it was determined that it displayed competitive inhibition. The 22-mer was further evaluated for secondary structures by MFold.

In the secondary structure of the 22-mer, a hairpin loop and a linear sequence were seen and the sequence for that hairpin loop was synthesized into a 10-mer and the linear sequence was synthesized into an 11-mer. The 10- and 11-mers were evaluated for inhibition of β-lactamase I but neither potential inhibitor was as potent as the 22-mer. The 10-mer was a competitive inhibitor but requires over 15-fold increase in the amount of inhibitor used compared to the 22-mer and the 11-mer showed no inhibition.

Competitive inhibition of β-lactamase I from *B. cereus* 569/H/9 by the 22-mer is beneficial to future studies because the aptamer may be able to bind to other β-lactamases from different sources. Future studies will determine if the aptamer can be considered a broad spectrum β-lactamases inhibitor. The broad spectrum inhibition could enable the aptamer to inhibit β-lactamases that are currently inhibitor resistant and thus enable the

22-mer to become a beneficial clinical drug for the treatment of penicillin resistant bacteria.

Currently several aptamers are present in clinical trials and an aptamer has been approved for the treatment of macular degeneration. These aptamers have not been shown to trigger immune responses. The lowered K_i and high specificity and affinity of these aptamers, while maintaining low toxicity make the aptamers ideal candidates for therapeutic use (Stoltenburg, et al., 2007).

Therapeutic use of the 22-mer would be more beneficial then the use of current β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam due to the lower MIC value, most likely giving the 22-mer a lower therapeutic dose (Willey, et al., 2010). Since previous aptamers show little toxicity, the 22-mer most likely has a higher toxic dose than that of current inhibitors (Stoltenburg, et al., 2007). With a higher toxic dose and a lower therapeutic dose the aptamer would have a higher therapeutic index and be a safer therapeutic agent then current β-lactamase inhibitors. Use of the novel inhibitor 22-mer would be better than the use of current inhibitors because bacteria have already formed resistance against the commonly used inhibitors. In the presence of resistance genes, such inhibitors would be required in higher amounts to counteract the resistance. If higher amounts of inhibitor are required for the therapeutic dose the therapeutic index of the decreases and the safety of the inhibitors would decrease.

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