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Inhibition of the PriA and PriB Primosome Proteins of the Neisseria gonorrhoeae Replication Restart Pathway.

Inhibition of *Neisseria gonorrhoeae* Replication Restart Pathways

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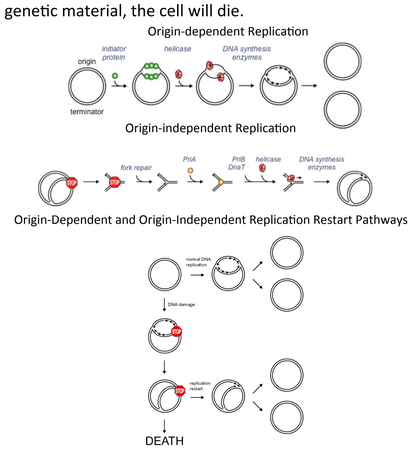
Abstract

DNA replication is essential to the survival of all living organisms. DNA damage that occurs in bacterial cells can lead to cell death by inhibiting the replication of genetic material. Bacteria, such as *Neisseria gonorrhoeae,* have developed a method to avoid cell death due to premature termination of DNA replication called a DNA replication restart pathway. Previous research has determined that there are two proteins, PriA and PriB, which play an important role in this pathway. Collectively these proteins bind to the DNA and act as enzymes, facilitating the reloading of the replication machinery and initiating replication without an origin sequence. This study explored possible inhibitors to the function of PriA and PriB and explored the method through which these inhibitors functions. The inhibitors studied could potentially be developed into novel antibiotics against *N. gonorrhoeae.*

Introduction

DNA replication is essential to the survival of all living organisms. DNA replication is catalyzed by a number of enzymes. A class of enzymes called helicases function to unwind and separate the double helix into two individual strands of genetic material. The replicative helicase binds to the double stranded DNA, unwinding it to create a "replication fork". DNA polymerases catalyze the addition of nucleotides to the individual strands of the DNA (1). This process will continue until the full chromosome is replicated, after which the DNA polymerase dissociates from the DNA leaving two full chromosomes. However, DNA damage can lead to termination of DNA synthesis before the whole chromosome is replicated (2). The termination of DNA synthesis often leads to the collapse of the replication fork, which then calls on repair pathways to perform their function to ensure that the chromosome is replicated (3). If a cell is not able to replicate its genetic material, the cell will die.



Hypothesis

A compound that inhibits the function of PriA and PriB will inhibit the replication restart pathway and will cause the bacterial cell to die due to its inability to fully replicate its DNA.

Specific Aims

- ✧ Identify an inhibitor to PriA and PriB proteins of the replication restart pathway in *N. gonorrhoeae*
- ✧ Determine the mode through which this inhibitor is functioning

Materials and Methods

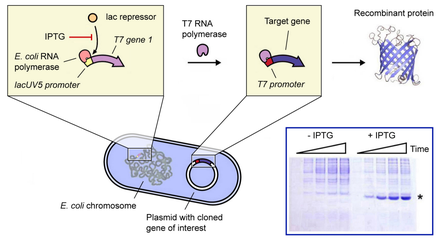
Generation of Reagents
- ✧ Purification of *N. gonorrhoeae* PriA and PriB proteins
- ✧ Construction of DNA substrate

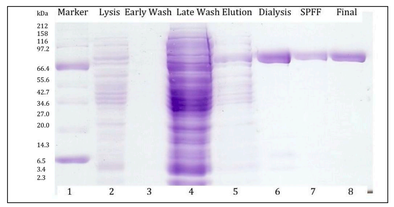
Discovery of Lead Compounds and Validation
- ✧ Helicase Assay
- ✧ High Throughput screening

Characterization of Lead Compounds
- ✧ Equilibrium Binding Assay
- ✧ ATPase Assay

Generation of Reagents

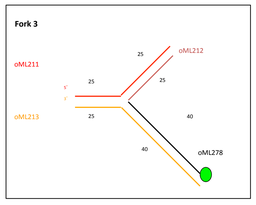
Purification of PriA and PriB from *E. coli* cells





SDS-page 10% Gel of Sequential steps of PriA preparation.
This figure shows the isolation of PriA through the sequential steps of the PriA preparation process. The first column is the marker then the next columns are samples taken from the following processes, in order: lysis of the cells, flow through of the bead/protein slurry (showing little to no protein), early wash with lysis buffer, late wash with lysis buffer, elution buffer wash, SPFF input, and final sample.

Construction of DNA substrate

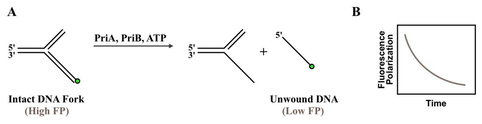


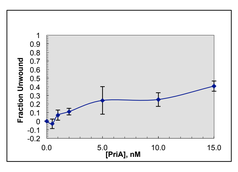
Fork 3 DNA substrate was prepared for use in in the Helicase assays

Discovery of Lead Compounds and Validation

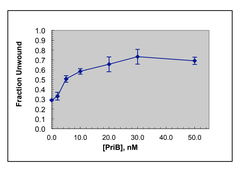
Helicase Assay and High Throughput Screening

This functional assay was performed to demonstrate the ability of PriA and PriB to unwind the fluorescent-tagged Fork 3 synthetic DNA substrate that acts like a replication fork, attracting the PriA and PriB to aid in unwinding and the amount of unwinding was be measured using spectroscopy.





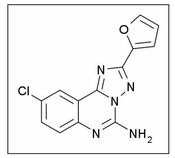
Helicase Assay Results with a varying concentration of PriA and no PriB.
This figure shows the ability of PriA to function as a helicase enzyme and unwind Fork 3 DNA even in the absence of PriB. PriA achieves approximately 40% unwinding of the Fork 3 DNA substrate at the low concentration of 15.0 nM.



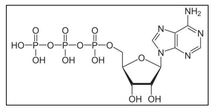
Helicase Assay Results with [PriA]= 2 nM and a varying concentration of PriB.
This figure shows that PriB and PriA together achieved significant unwinding of the Fork 3 DNA substrate. At concentrations as low as 10 nM PriB, 60 % unwinding was seen and a maximum of close to 75 % unwinding was seen in the absence of inhibitor.

High Throughput Screening

CGS-15943



Adenosine triphosphate (ATP)



Helicase assays were used as the basis for the High Throughput Screening performed at the University of Wisconsin.

The higher the helicase activity of the protein the less polarized the light is

CGS-15943 was chosen to pursue as a potential inhibitor based on the results of the high throughput screening. It was originally hypothesized that CGS-15943 would be a competitive inhibitor to the ATPase activity of the PriA and PriB proteins due to its structural similarities to ATP (shown above). Both ATP and CGS-15943 contain an adenine base attached to a five-membered ring containing one oxygen.

Characterization of Lead Compounds

Equilibrium Binding Assay

This functional assay was performed to demonstrate the ability of PriA to bind to Fork 1 DNA and the ability of PriB to bind to a single stranded DNA oligo (ssDNA oML228) using fluorescence polarization spectroscopy to test protein-DNA interactions. This assay measured the rates of rotational diffusion, or tumbling, as a read-out of complex formations, where small molecules have low polarization because they tumble slower than larger molecules, which have higher polarization.

Equilibrium Binding Assay – PriA with Fork 1

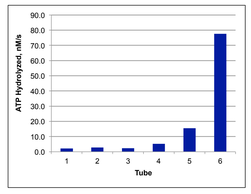
Average K_d	15.3 ± 4.7 nM

Equilibrium Binding Assay – PriB with ssDNA oML228

Average K_d	479.1 ± 68.9 nM

ATPase Assay

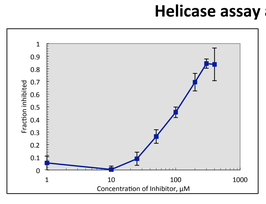
This functional assay was performed to demonstrate the ability of PriA and PriB to catalyze the hydrolysis of ATP. This assay used an ATP regeneration system that converts ADP to ATP in a reaction that is coupled to the conversion of NADH to NAD^+. The coupled reaction was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm due to NADH oxidation. This spectrophotometric assay was used to measure the kinetic parameters V_{max} and K_m in the presence and absence of the inhibitor compounds to see if the inhibitors affect PriA-catalyzed ATP hydrolysis.



ATPase Assay without Inhibitor.
This figure shows the rate of ATP hydrolysis with various components. Tube 1 contained the master solution, NADH, DNA, and ATP only; tubes 2 and 3 lacked PriA and PriB, respectively, and contained no DNA; tube 4 contained both PriA and PriB and lacked DNA; tube 5 lacked only PriB; and tube 6 contained all the components in tube one plus PriA and PriB.

Results

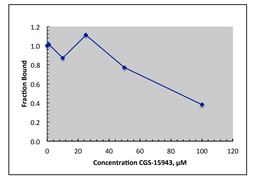
Helicase assay and IC_{50} Determination



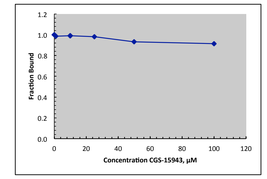
Helicase Assay Results in the presence of Inhibitor.
This figure shows the fraction PriA and PriB proteins' helicase activity that was inhibited in the presence of increasing amounts of inhibitor. The [PriA] was 5 nM and the [PriB] was 50 nM. Significant inhibition is seen with as little as 100 µM CGS-15943. This data was used to determine the IC_{50} value.

Helicase Assays were performed in the presence of inhibitors to determine the IC_{50} concentration of the CGS-15943 inhibitor which was then used in the additional functional assays to determine the mode of inhibition.

Equilibrium Binding Assay

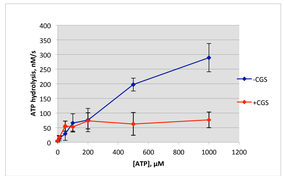


Equilibrium Binding Assay of PriA with Fork 1 DNA in the presence of Inhibitor.
This figure shows the significant inhibition observed when the CGS 15943 compound was added to the Equilibrium binding assay of PriA with Fork 1 DNA. At concentrations of inhibitor as low as 80 µM close to 50% of the equilibrium binding achieved at the K_d concentration is inhibited.



Equilibrium Binding Assay of PriB with ssDNA substrate oML 228 in the presence of Inhibitor.
This figure shows that the CGS 15943 inhibitor had little to no effect on the ability of PriB to bind to oML 228 DNA oligo. Even at concentrations of the inhibitor where only 40 % of the PriA was bound to Fork 1 DNA close to 100% of PriB is bound to oML 228.

ATPase Assay Results



: Effects of Inhibitor on ATP Hydrolysis.
This figure shows the decrease in V_{max} that the CGS-15943 compound had on the rate of ATP hydrolysis.

	Km	Vmax
No Inhibitor	91.47 ± 36.15	196.03 ± 7.04
Inhibitor	142.02 ± 124.06	68.72 ± 23.02

Effect of the Inhibitor on the Kinetic Parameters of the ATPase enzyme associated with the replication restart pathway of *N. gonorrhoeae.*
This figure shows the inhibitor effecting both the V_{max} and the K_m values. The decrease in V_{max} was significant however the change in K_m was inconclusive suggesting either a mixed or an uncompetitive inhibitor.

Conclusions

✧CGS 15943 functions as an inhibitor to the helicase activity of PriA and PriB which was shown in the Helicase assays and IC_{50} determination.

✧CGS 15943 affects PriA's ability to bind to DNA while having little to no effect on PriB's DNA binding which was shown in the Equilibrium binding assay

✧CGS 15943 is not a competitive inhibitor based on the ATPase results
- ✧ CGS 15943 is either a mixed or uncompetitive inhibitor – it either binds to the enzyme on a site distinct from the active site, binds to the enzyme – substrate complex, or both

Future Work

✧Determine if CGS-15943 inhibits the PriA of bacteria other than *N. gonorrhoeae*.
✧ Optimize the potency of CGS-15943 by substituting chemical functional groups.
✧Determine the effect of CGS-15943 on bacterial cell growth and mammalian cell growths.

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