OVEREXPRESSION, PURIFICATION, AND INHIBITION OF HELICOBACTER PYLORI ALDO-KETO REDUCTASE (HPAKR) USING DESIGNER INHIBITORS

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ABSTRACT: Helicobacter pylori infects the gastric mucosa of over half of the world's population, and is implicated in the genesis of many gastric pathologies. Current treatments for H. pylori infections are becoming increasingly ineffective as antibiotic-resistant strains of H. pylori become more prevalent. The purpose of this project is to discover a competitive inhibitor for an aldo-keto reductase enzyme (HpAKR) that is required for H. pylori to survive in the human stomach. The plasmid containing the HpAKR gene will be transformed into Rosetta™ E. coli cells (Novagen) for overexpression, and the purified HpAKR molecules will be assayed for activity and inhibition.

Helicobacter pylori, a bacterium which primarily colonizes the gastric mucosa, infects over half of the world's population, and is one of the most common agents of chronic bacterial infection in humans (Garcia, Salas-Jara, Herrera, & Gonzalez, 2014; Krah et al., 2004). Although most infected individuals are asymptomatic, *H. pylori* infections are associated with the majority of gastric pathologies, particularly chronic gastritis and gastric and duodenal ulceration (Garcia et al., 2014). It is estimated that 90-95% of duodenal ulcers in Europe are due to *H. pylori* infections (Krah et al., 2004). Additionally, *H. pylori* has been classified by the World Health Organization (WHO) as a Class I carcinogen (Ernst & Gold, 2000), and *H. pylori* infections have been associated with a 2.7– to 12–fold increase in the risk of developing gastric cancer (Krah et al., 2004).

Socioeconomic status (SES) plays a pivotal role in determining the risk of contracting an *H. pylori* infection (Malaty & Graham, 1994). This is particularly evident in developing countries where infection rates can be as high as 90%. (Garcia et al., 2014). A study conducted by Malaty and Graham (1994) on the prevalence of *H. pylori* infections among individuals living in the Houston metropolitan area found that 85% of individuals in the lowest social classes were infected with *H. pylori* versus only 11% of individuals in the top two social classes. The correlation between low SES and higher rates of *H. pylori* infection is due to a variety of factors often associated with low SES including poor hygiene and overcrowded living conditions (Laszewicz, Iwańczak, & Iwańczak, 2014).

H. pylori infections are currently treated using antibiotics and a proton pump inhibitor, however, such treatments are becoming increasingly ineffective due to the evolution of antibiotic-resistant *H. pylori* strains (Megraud, 2004). Antibiotic resistance rates vary depending on geographical location, but resistance rates for clarithromycin and metronidazole, two of the antibiotics most commonly used to treat *H. pylori* infections, have been reported to be as high as 25% and 76%, respectively (Megraud, 2004). Although other antibiotics such as tetracycline or amoxicillin may also effectively treat *H. pylori* infections, the current trend of increasing resistance rates for the common antibiotics may eventually render these antibiotic options equally ineffective (Megraud, 2004). Thus, the development of alternative treatment methods for *H. pylori* infections is paramount.

The key to discovering an alternative treatment lies in understanding how *H. pylori* is able to survive the harsh acidic conditions of the human stomach. Initial studies indicate that *H. pylori* colonization is facilitated by the use of urease, an enzyme capable of converting urea into ammonia and bicarbonate (Marshall, Barrett, Prakash, McCallum, & Guerrant, 1990). As shown in the reaction below, urease removes H^+ ions from the bacterium's immediate environment in order to create ammonia and bicarbonate. This reaction neutralizes the H^+ ions present in the gastric juice, thereby increasing the local pH (Marshall et al., 1990). However, urease-negative *H. pylori* strains are still able to colonize the gastric mucosa, indicating that other factors are required for *H. pylori* to survive in acidic environments (Mine, Muraoka, Saika, & Kobayashi, 2005).

$$(NH2)2CO + 2H2O + H^+ \xrightarrow{\text{Urease}} 2NH4^+ + HCO3^-$$

A recent study by Bijlmsa et al. (2000) identified ten different genes whose products may enable *H. pylor*i to survive in acidic conditions. One of these genes codes for an aldo-keto reductase (HpAKR). HpAKR functions over a broad pH range (pH 4-9), but displays optimum activity levels at a pH of 5.5, which is similar to the pH of the gastric mucosa of the human stomach (Cornally et al., 2008). Although the exact function of HpAKR is unknown, it appears that the enzyme is required for growth at a low pH. Isogenic mutants—mutants with nearly the same genotype as wild type—of *H. pylori* without the HpAKR gene were unable to grow at a pH of 5.5 (Cornally et al., 2008). Given that HpAKR is necessary for the pathogenesis of *H. pylori* within the human stomach, it serves as a promising target for pharmaceutical development. This particular study describes the expression, purification, kinetics, and inhibition of HpAKR with the hope of finding a competitive inhibitor for the enzyme.

Methods And Materials

Plasmid Preparation of HpAKR/pET28b

In order to create the necessary amount of HpAKR enzyme, the pET28b plasmid (which harbors the HpAKR gene) will be transformed into DH5α, a storage strain of *E. coli*. The DH5α cells will be grown on Lennox Broth (LB) agar plates containing 50 mg/mL Kanamycin (KAN) and 34 mg/mL Chloramphenicol (CAM) (antibiotics used to prevent other types of bacteria from growing on the plates). One of the DH5α colonies will be used to start an *E. coli* culture containing the pET28b/HpAKR plasmid and will be grown in 5 mL of LB containing 5 µL of KAN (50 mg/mL) and 5 µL of CAM (34 mg/mL). The culture will shake in the Excella™ E24 Incubator (New Brunswick Scientific) at 180 rpm and 37°C for approximately 15 hours. Following incubation, the pET28b/HpAKR plasmid will be purified using a Qiagen plasmid purification kit and then transformed into Rosetta™ *E. coli* cells (Novagen) for overexpression.

HpAKR Overexpression

To overexpress the HpAKR plasmid, transformed Rosetta™ cells will be grown at 37°C in 1 Liter of LB broth with 5 µL of KAN (50 mg/mL) and 5 µL of CAM (34 mg/mL). A UV-Vis Spectrometer (BioMate) will be used to measure the absorbance of the cells at 600 nm. When the absorbance value is between 0.80 and 1.00, the cells will be induced with 1M IPTG (final concentration 1mM) and grown at 37°C for approximately 15 hours. The cells will be isolated via centrifugation at 5000 x g for 10 minutes (Beckman Coulter, JA17 rotor). Following centrifugation, the cell pellet will be re-suspended in a 25mL Lysis buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole. The cells will be sonicated for five 30-second intervals with a 1 minute rest period in between each sonication period. The sonication step will lyse the cells to release all of the HpAKR molecules produced during

the overexpression phase. Cell debris will be removed via centrifugation at 15,000 x g for 20 minutes.

The supernatant will be loaded onto a Nickel-Nitriloacetic Acid resin column (Novagen) to separate HpAKR from any other proteins present in the supernatant. The column will be washed five times with Wash Buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 30 mM imidazole to remove the undesired proteins. HpAKR will be eluted from the column using Elution Buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 150 mM imidazole. The eluted fractions will spin in the centrifuge twice, first for 20 minutes at 3500 RPM, then at 4000 RPM for 30 min with 7 mL of the Lysis buffer. Following the final centrifugation, the sample will contain pure HpAKR, which can be used to run activity and inhibition assays. The purity of HpAKR will be evaluated using 12%-SDS-PAGE.

Activity Assays for HpAKR

Following the literature procedures (Cornally et al., 2008), the UV-Vis spectrophotometer will be used to monitor the reduction of benzaldehyde to benzyl alcohol by NADPH at 340nm. The assay (total volume 1 mL) will consist of 50 mM potassium phosphate buffer, 500 mM NADPH, 10 µM HpAKR, and benzaldehyde concentrations ranging from 0.5 mM to 20 mM. The molar extinction coefficient (ε) that will be used for NADPH is ε_{340}= 6.2 x 10^3 $M^{-1}cm^{-1}$. K_M and k_{cat} for the purified HpAKR will be determined by fitting initial rates to the Michaelis-Menten equation and by using the linear regression of a Lineweaver-Burke plot in Microsoft Excel™. If the calculated K_M and k_{cat} are similar to literature values (Mine et al., 2005), then the HpAKR molecules produced during the overexpression and purification phase are sufficiently active and can be used in the inhibition assays.

Inhibition of HpAKR

Potential inhibitors for HpAKR will be determined by Dr. Peter Anderson (University of Washington Bothell) using Autodock Vina (Trott, & Olson, 2010), a program designed determine the binding affinity (i.e., docking ability) of potential inhibitors to a particular enzyme. Dr. Anderson will screen thousands of potential inhibitors and recommend at least 10 potential inhibitor molecules with K_i values (binding affinity values) between 15 and 200nM. Two of these inhibitors will be selected and used for inhibition assays.

Similar to the activity assays, the UV-Vis spectrophotometer will be used to monitor the reduction of benzaldehyde to benzyl alcohol by NADPH at 340nm. The assay for each inhibitor (total volume 1 mL) will contain 50 mM potassium phosphate buffer, 500 mM NADPH, 10 µM HpAKR, benzaldehyde concentrations ranging from 0.5 mM to 20 mM, and inhibitor concentrations ranging from 0 to 5 µM. Kinetic parameters (K_M and k_{cat}) for HpAKR will be computed by fitting initial rates to the Michaelis-Menten equation and using the linear regression of a Lineweaver-Burke plot in Microsoft Excel™.

The shape of the Lineweaver-Burke plots will indicate whether the inhibitors are competitive, non-competitive, or mixed inhibitors (Figure 1). Ultimately, we hope to find a competitive inhibitor for HpAKR since a competitive inhibitor will only interact with HpAKR while a non-competitive or uncompetitive inhibitor may interact with other proteins present in the sample.

The methodologies described above are commonly used in protein purification and the kinetic analysis of proteins and can easily be completed by undergraduate students under the advisement of a faculty mentor. A list of required materials can be found in the Appendix.

Objectives and Deliverables

Objective 1. Express and purify HpAKR. The deliverables for this objective will include a

photograph of the SDS-PAGE gel showing the transformed DNA plasmid with the HpAKR gene, a photograph of the SDS-PAGE gel showing the purification of the HpAKR, and a fully detailed protocol for the expression and purification process.

Objective 2. Run activity assays using the purified HpAKR. The deliverables for this objective include a data table showing the raw absorbance vs. time data and kinetic scan plots of the absorbance vs. time data, as well as detailed calculations for determining reaction velocity values and substrate concentrations used in the assay. Additionally, we shall create Michaelis-Menten and Lineweaver-Burke plots using the reaction velocity data and use the equation generated by the Lineweaver-Burke plot to calculate K_M and k_{cat}. We will also write a fully detailed protocol for the activity assays.

Objective 3. Run inhibition assays using Inhibitor 1 and Inhibitor 2. The deliverables for this objective will be the same as the deliverables for Objective 2, including a fully detailed protocol for the inhibition assays.

Broader Impacts

The primary focus of our education and outreach efforts will be teaching our fellow undergraduate students from all disciplines about the importance of finding alternative methods to treat *H. pylori* infections. First, the members of the HpAKR project will create and present a brief summary of our findings along with any relevant background information to the other members in Dr. Robins' research lab. This presentation will inform our colleagues on the status of our project as well as increase their knowledge on the topic. Additionally, the HpAKR project members will create a poster which will be presented at the University of Washington Bothell's Undergraduate Research Fair in the spring of 2016. This will allow us to share the knowledge gained from the project to other undergraduate students who conduct research in a wide range of disciplines.

In order to foster an interest in biochemical research in first and second year students at UW Bothell, the HpAKR team members will partner with the on-campus entities such as the Office of Undergraduate Research and the Pre-Medical Club at the University of Washington Bothell. These entities will help to recruit new students to conduct further research in Dr. Robins' lab. Furthermore, the HpAKR team members will hold several information sessions throughout Winter Quarter. These sessions will provide interested students with background information about the current state of the project and answer the students' questions regarding our experiences working within the lab. The current HpAKR project members will also assist in teaching the new research students the skills and techniques necessary to conduct further research on HpAKR.

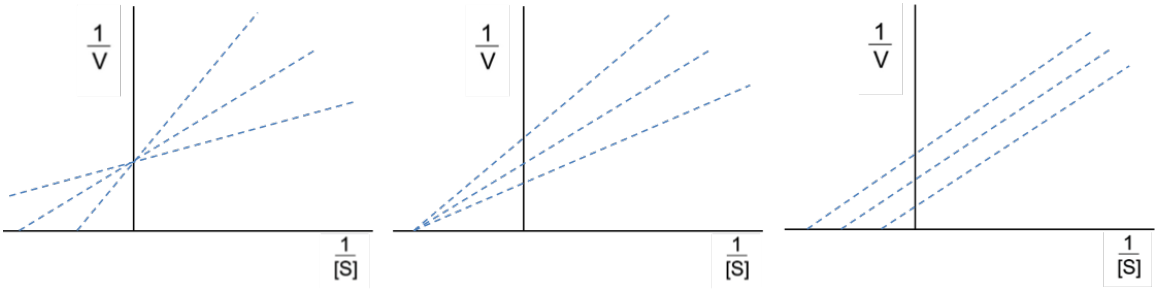

Figure 1: Lineweaver-Burke Plots for Competitive inhibition (left), Non-competitive inhibition (center) and Uncompetitive inhibition (right).

With the assistance of Dr. Robins, the HpAKR project members will write and publish a paper detailing our methodologies and findings in order to disseminate what we have learned to other scientists who are working with *H. pylori*. If we are successful in finding an effective competitive inhibitor for HpAKR, researchers in the drug development field can explore the feasibility of incorporating the inhibitor into a treatment for individuals who are infected with *H. pylori*. If we find that the inhibitors are not competitive in nature or do not effectively inhibit HpAKR, then future researchers will know to either test other potential inhibitors or to improve upon the method we used to test our inhibitors. Ultimately, our research will provide current and future *H. pylori* researchers with new insight into how to resolve the *H. pylori* predicament.

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Appendix

Required Materials

Instruments	Glassware, Containers, and Pipettes	Reagents and Cells	Miscellaneous
New Brunswick Scientific Excella E24 Incubator Shaker Series	125 mL, 200 mL, and 500 mL Pyrex Erlenmeyer Flasks	DH5α cells	Fisher Scientific 3mm solid glass beads
BioMate 3s UV-Vis Spectrometer	0.2-20 µL, 0.5-10 µL, 20-200 µL, and 100-1000 µL micropipettes with tips	Lennox Broth (Fisher Bioreagents®)	Qiagen Plasmid Purification Kit
BioRad Gel Doc EZ Imager	Microcentrifuge tubes	Agar pellets (Fisher Bioreagents®)	Nickel-Nitrolotriaectic Acid resin column (Novagen)
Avanti® J-E Centrifuge by Beckman Coulter	Large centrifuge tubes	Kanamycin and Chloramphenicol (Fisher Bioreagents®)	Quartz cuvettes
BioRad Electrophoresis Kit	Glass test tubes with lids and test tube rack	Novagen Rosetta® Singles Kit	
Microfuge® 20R Centrifuge by Beckman Coulter	100 mL Pyrex Beaker	Tris-Borate-EDTA (TBE) (Fisher Bioreagents®)	
Fischer Scientific Vortex Mixer	Filter tube	1M IPTG (Fisher Bioreagents®)	
Fisher Scientific Isotemp® Refrigerator		Nanopure water from NANOpure Infinity	
Autoclave		Lysis buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 5 mM imidazole]	
		Wash Buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 30 mM imidazole]	
		Elution Buffer [20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, and 150 mM imidazole]	
		Benzaldehyde stock (200 mM)	
		NADPH stock (500mM)	
		Potassium phosphate buffer (50 mM)	
		Inhibitors (eMolecule)	