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The Discovery and Development of Thienopyrimidines as Inhibitors of *Helicobacter pylori*

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Abstract

The rate of successful treatment for *Helicobacter pylori* infections, with the clarithromycin triple therapy, is only 75%. The triple therapy, which consists of a proton pump inhibitor and two broad-spectrum antibiotics such as clarithromycin and amoxicillin, is becoming less effective due to the rise of strains with resistance against these antibiotics. In the search for narrow spectrum drugs for the treatment of *H. pylori* infections, a high-throughput screen was performed to identify selective compounds against *H. pylori*. This screen revealed two selective and structurally related thienopyrimidines. Structure-activity relationship of the thienopyrimidines against *H. pylori* was examined through the synthesis of 27 analogs, efforts that merged elements of the two scaffolds. The resulting leads, including compound 25, demonstrate high potency with an acceptable cytotoxicity profile against the human FaDu cells. Mode of action studies were performed by the generation and sequencing of resistant mutants. These experiments identified *H. pylori*'s respiratory Complex I as the putative target of the series, with the amino acid changes found in the NuoD subunit. Lead compounds demonstrated efficacy in an ex vivo model but not in the in vivo studies in a *H. pylori* murine infection model, suggesting further optimization of the pharmacological properties is required for this series. A scaffold hopping exercise was performed to discover novel scaffolds with improved ADME properties. The pyrrolopyrimidine 56 emerged with good anti-*H. pylori* activity, an improved solubility and is less bound to human plasma protein. Lead optimization efforts are ongoing, to realize proof of concept of this series of compounds in the in vivo efficacy studies.

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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTOR OF PHILOSOPHY DISSERTATION

**The Discovery and Development of
Thienopyrimidines as Inhibitors of *Helicobacter
pylori***

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in

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DEDICATION

To my parents, Gorrette and Celestin,
For their unconditional love and encouragement.

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ABSTRACT

The rate of successful treatment for *Helicobacter pylori* infections, with the clarithromycin triple therapy, is only 75%. The triple therapy, which consists of a proton pump inhibitor and two broad-spectrum antibiotics such as clarithromycin and amoxicillin, is becoming less effective due to the rise of strains with resistance against these antibiotics. In the search for narrow spectrum drugs for the treatment of *H. pylori* infections, a high-throughput screen was performed to identify selective compounds against *H. pylori*. This screen revealed two selective and structurally related thienopyrimidines. Structure-activity relationship of the thienopyrimidines against *H. pylori* was examined through the synthesis of 27 analogs, efforts that merged elements of the two scaffolds. The resulting leads, including compound **25**, demonstrate high potency with an acceptable cytotoxicity profile against the human FaDu cells. Mode of action studies were performed by the generation and sequencing of resistant mutants. These experiments identified *H. pylori*'s respiratory Complex I as the putative target of the series, with the amino acid changes found in the NuoD subunit. Lead compounds demonstrated efficacy in an *ex vivo* model but not in the *in vivo* studies in a *H. pylori* murine infection model, suggesting further optimization of the pharmacological properties is required for this series. A scaffold hopping exercise was performed to discover novel scaffolds with improved ADME properties. The pyrrolopyrimidine **56** emerged with good anti-*H. pylori* activity, an improved solubility and is less bound to human plasma protein. Lead optimization efforts are ongoing, to realize proof of concept of this series of compounds in the *in vivo* efficacy studies.

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CHAPTER 1. INTRODUCTION

H. pylori Infections and Diseases

Helicobacter pylori infects more than 50% of the world's population.^{1,2} In the United States, more than 500 000 cases of *H. pylori* infections are diagnosed annually, according to the Center for Disease Control and Prevention.³ The majority *H. pylori* infections leads to gastritis, often without showing symptoms.^{1,4} 10% of the *H. pylori* infections lead to peptic ulcers.⁵ Peptic ulcers are often chronic and may cause bleeding, perforation and eventually death.⁴ Less commonly, *H. pylori* infections may result into certain forms of cancer such as mucosa-associated lymphoid tissue (MALT) lymphoma, gastric and pancreatic adenocarcinoma.⁶ The World Health Organization (WHO) lists *H. pylori* as a class I carcinogen.⁷ Often, acute gastritis becomes chronic and then chronic gastritis leads to metaplasia and adenocarcinoma.⁴ Gastric cancer is the fourth most common cancer and the second leading cause of deaths among all cancers.⁸ *H. pylori* has also been implicated in the pathogenesis of many other diseases including idiopathic thrombocytopenia purpura, idiopathic iron deficiency anemia, ischaemic heart diseases, ischaemic cerebrovascular disease, atherosclerosis, Raynaud's phenomenon and many skin diseases.⁴

Barry Marshall and Robin Warren were awarded the Nobel Prize in 2005, for their work that established *H. pylori* as the causative agent of gastritis and peptic ulcers.⁹ Their discovery enlightened the long-standing ambiguity around the cause of peptic ulcers. As result, patients with peptic ulcers and gastric cancer are recommended to be tested for *H. pylori* infections.¹⁰ The tests that are conducted to identify active infection include the urea breath test, fecal antigen test and mucosal biopsy-based testing that relies on endoscopy.¹¹ *H. pylori* is generally identified in 70% of antral biopsy specimen of patients with chronic gastritis and 90% of patients with duodenal ulcers.⁴ The eradication of *H. pylori* is recommended in treating gastritis, instead of drugs that suppress the high gastric acidity.^{12,13,14} Patients treated by eradicating the bacteria show lower relapse rate (9%) without recurrence, as oppose to high relapse rates (58-67%) identified with patients who manifest the infection after treatment.⁴ *H. pylori* eradication is also recommended in both treating low grade MALT lymphoma and preventing gastric cancer.¹⁵⁻¹⁷ According to a study that was conducted in Japan, nearly 80% of 420 patients who tested positive for the gastric MALT lymphoma, responded to the eradication therapy with minimal residual disease.¹⁸ Usually, the eradication of *H. pylori* is confirmed using the tests, mentioned above.¹¹

The Treatment Against *H. pylori* Infections

The clarithromycin triple therapy consists of a Proton Pump Inhibitor (PPI), clarithromycin and either amoxicillin or metronidazole for 14 days. (**Figure 1-1**) In the 2017 American College of Gastroenterology (ACG) guidelines, the clarithromycin triple therapy is recommended as the first-line treatment for *H. pylori* infections.¹¹ However,

Figure 1-1. Anti-*H. pylori* drugs.

(A) Structure of clarithromycin. (B) Structure of amoxicillin. (C) Structure of metronidazole. (D) Structure of levofloxacin. (E) Structure of ciprofloxacin. (F) Structure of rifabutin.

the clarithromycin triple therapy have shown limitations. The clarithromycin triple therapy has been associated with lower eradication rates, when it is administered for a shorter period of 7 to 10 days. The drug, clarithromycin is highly recommended as a first line treatment for patients who have not been previously exposed to macrolides and who are in the area identified with low resistance against clarithromycin (< 15%).¹⁹ Clarithromycin is a bacterial static agent that acts following the inhibition of protein synthesis via binding to the 50S ribosomal subunit. Amoxicillin is a bactericidal agent and inhibits the synthesis of the cell wall. These antibiotics act on actively replicating bacteria.²⁰ A proton pump inhibitor such as omeprazole, acts by suppressing the secretion of gastric acid, which allow the bacteria to replicate and facilitate the bactericidal effects of the antibiotics.

A strong association has been reported between the number of previous exposures and a high risk of antibiotic resistance.²¹ In general, the appropriate regimen for the treatment against *H. pylori* infections is recommended based on the drug resistance pattern in the region and the patient's exposure to the antibiotics and the patient compliance.²² For patients who have an allergy to penicillin, amoxicillin is substituted with metronidazole in the clarithromycin triple therapy regimen. The use of either metronidazole or amoxicillin achieves the same therapeutic effect.²³ Inside the bacteria, metronidazole is metabolized by nitroreductase enzymes into metabolites that are toxic to bacteria. The triple therapy regimen, containing metronidazole, is recommended to patients who have been previously exposed to macrolides.

The bismuth quadruple therapy is another recommended first-line treatment, which consists of a PPI, bismuth, a tetracycline and a nitroimidazole for 10-14 days.¹¹ **(Figure 1-1)** In case the clarithromycin triple therapy fails to eradicate the infection, the bismuth quadruple therapy is recommended as a salvage treatment. The use of bismuth as part of the regimen achieves the eradication rate of above 90%.²⁴ However, the role of bismuth is not well understood. Marcus et al. suggests that bismuth hinders proton entry into the bacteria which leads to the up-regulation of growth depended genes and *H. pylori* becomes more susceptible to the antibiotics.²⁵

The concomitant, the sequential, the hybrid and the fluoroquinolone sequential therapies are other first line treatment options that are used worldwide for the treatment of *H. pylori* infection. The concomitant therapy consists of a PPI, clarithromycin, amoxicillin and a nitroimidazole for 10 to 14 days.¹¹ The sequential therapy consists of a PPI and amoxicillin for 5 to 7 days followed by a PPI, amoxicillin, clarithromycin and a nitroimidazole for 5 to 7 days. The hybrid therapy consists of a PPI and amoxicillin for 7 days followed by a PPI, amoxicillin, clarithromycin and a nitroimidazole for 7 days. Fluoroquinolone sequential therapy consists of a PPI and amoxicillin for 5-7 days followed by a PPI, fluoroquinolone and nitroimidazole for 5-7 days with levofloxacin and ciprofloxacin as the fluoroquinolone drugs. **(Figure 1-1)** The concomitant, the sequential and the hybrid therapies have been found to achieve the same efficacy based on clinical research data published by ACG.¹¹ According to the recent meta-analysis, the sequential and concomitant therapies have shown higher eradication rates (>90%), than the clarithromycin triple therapy (>70%).¹⁰ The concomitant therapy, in particular, is

preferred among these alternative first-line therapies, given it constitutes a simpler regimen that affords patient compliance.

The levofloxacin triple therapy, the rifabutin triple therapy and the high-dose dual therapy are recommended as salvage treatments, in case the first line treatment fails to eradicate the infections. **(Figure 1-1)** The levofloxacin triple therapy, which consists of a Proton Pump Inhibitor (PPI), levofloxacin and amoxicillin for 10 to 14 days, is recommended as a salvage regimen, in case the clarithromycin triple therapy or the bismuth quadruple therapy fails to eradicate *H. pylori*. Rifabutin triple regimen consists of a PPI, amoxicillin and rifabutin for 10 days. High-dose dual therapy consists of a PPI and amoxicillin administered for 14 days. Despite the availability of these alternative regimens, the rate of successful treatments has significantly declined worldwide, due to the rise of resistance against the antibiotic components of these regimens.^{1,26}

The Resistance Against Anti-*H. pylori* Drugs

H. pylori has become a global threat due to a steady increase of worldwide drug resistance against anti-*H. pylori* drugs. *H. pylori* is listed as a high priority drug-resistant bacterium by the World Health Organization among bacteria that are threat to the human health.⁷ Worth noting, the rate of successful treatment of *H. pylori* infections has dropped below 80%.^{1,26} In a recent study, 22% of patients tested in a clinical trial were not cured by either the triple therapy or the quadruple therapy.²⁷ In the early 1990s, the worldwide treatment rate of *H. pylori* infections with the triple therapy were recorded at 80-90%.²⁸ From 1998 to 2002, the United States national sampling study reported a resistance rate of 21% against metronidazole and 13% against clarithromycin.²⁹ Between the year of 2000 and 2008, the resistance rate in Alaska was recorded at 42% and 30% against metronidazole and clarithromycin respectively.³⁰ In 2012, the resistance rates were reported at 20% and 16% by the Houston VA medical center, for metronidazole and clarithromycin respectively.³¹ In particular, the resistance against metronidazole has been fueled by its overuse in the regions predominated by parasitic diseases.¹⁹ In certain parts of the world, such as Japan, the resistance rate against metronidazole is low, due to its restricted use at the national level.³²

The effectiveness of the regimens for the treatment of *H. pylori* infection, has declined due to the resistance against the antibiotics at various degrees. The meta-analysis studies have revealed that the resistance against clarithromycin is the major contributor to the failure of the triple therapy.¹⁰ More worrisome, the fluoroquinolone resistance has been recorded as high as clarithromycin resistance (rate: 16%), in North America.^{31,33} Although *in vitro* resistance against metronidazole (rate: 20%) is high worldwide, *H. pylori* mutations against clarithromycin or levofloxacin are the most clinically relevant mutations. They are usually identified using molecular methods including polymerase chain reaction or fluorescently-labeled nucleic acid hybridization. These methods have been optimized overtime and are simply applied to clinical isolates collected from gastric mucosal biopsies or fecal samples.³⁴ The resistance against amoxicillin, tetracycline and rifabutin are still low (rate: < 2% for each), whereas the

resistance against bismuth hasn't been reported worldwide.^{11,35} The resistance rates against these antibiotics are registered higher in children than adults, because the same drugs are prescribed for the treatment of diseases that are prevalent in children, such as the respiratory and parasitic diseases.³⁶ Clarithromycin, fluoroquinolones and rifabutin are generally discouraged to be reused in the clinic, given the resistance against these drugs cannot be overcome by simply increasing the dose.

Point mutations have been the primary mechanism of resistance against the antibiotics for the treatment of *H. pylori* infections. In *H. pylori*, point mutation in the 23S rRNA are the main mechanism of resistance against clarithromycin. Mutations in the penicillin binding proteins drive the resistance against amoxicillin. The resistance against levofloxacin comes from the mutation in the *gyrA* gene, which encodes the subunit A of DNA gyrase.³⁸ Various mechanisms have been reported as the basis for the resistance against metronidazole.

The resistance against metronidazole originates from the mutations in the *rdxA* gene. In bacteria, *rdxA* encodes for NADPH nitroreductase, which is responsible for activating metronidazole and other nitroaromatic compounds into toxic metabolites.³⁹ The efflux pump, *hefA* is found overexpressed in resistant *H. pylori* strains and has been reported to play a key role in reducing the cellular concentrations of metronidazole.⁴⁰ *hefC*, a multidrug efflux pump, also reduces drug concentrations to below effective concentrations.⁴¹

The side effects from the use combination broad spectrum antibiotics, contributes to poor patient compliance affecting the success of these treatments.¹⁰ The use of these broad-spectrum antibiotics perturbs the gut microbiome, leading to symptoms like diarrhea.¹¹ Other side effects associated with the triple therapy include: headache, dizziness, nausea, dysgeusia, dyspepsia, pseudomembranous colitis, mycosis, sore mouth and tongue, drug hypersensitivity and paraesthesia. Given the rise of resistance, specific therapies against *H. pylori* infections are needed to preserve these broad-spectrum drugs, that are crucial for the treatment of other infections and to mitigate the side effects associated with these drugs.

***H. pylori*'s Colonization and Virulence**

H. pylori is a spiral gram-negative bacterium that colonizes in the human stomach.⁴² *H. pylori* infections are generally acquired at a young age via oral-oral route.^{43,44,45} Given *H. pylori* can only survive for 30 minutes, *H. pylori* must move quickly to avoid gastric emptying and penetrate the stomach mucus layer.⁴⁶ *H. pylori* is found in the corpus and the antrum regions of the stomach, which differ in their cellular make up.⁴⁷ *H. pylori* colonizes areas in close association to the epithelial cells, to form a niche where it releases cytotoxins.⁴⁸ Although, the majority of bacteria are found freely swimming in the lumen, important populations of *H. pylori* are also found beneath the gastric mucous layer and within the glands, regions that are considered more favorable environment for long term survival than the lumen (pH < 2).

The pH gradient of different regions of the stomach is a major factor in determining *H. pylori*'s colonization site.⁴⁶ *H. pylori* is a bioenergetically neutrophil, with the ability to survive in an environment with a pH range of 4-8.⁴⁹ Unlike other bacteria, *H. pylori* can inhabit acidic milieu, by maintaining a favorable pH in the proximity via 2 proteins: the urease which hydrolyzes urea into CO₂ and ammonia, and the α -carbonic anhydrase protein, which catalyzes the conversion of CO₂ to HCO₃⁻ and eventually reduces the concentrations of HCl.⁵⁰⁻⁵² *UreI* is the proton gated channel, responsible for the urea influx. The protein urease is also found at the surface layer of the cell. The ammonia reacts with HCl to create a neutral surrounding environment in the periplasm and around the bacterium. The diffusion rate of chemicals, including ammonia, is significantly diminished in the mucus, which allows the acid to be neutralized and the neutral pH is maintained for a long period.⁵³

H. pylori reaches the epithelial cells at the surface of the stomach, by motility with the flagella and the chemotaxis signaling system.⁵⁴ Recent studies have shown that the wildtype strains swim faster in viscous environment than the mutants with straight cell bodies, suggesting *H. pylori*'s spiral shape facilitate the bacteria to penetrate the mucus, in addition to the flagella.^{55,56} The bacterium utilizes chemotaxis, a mechanism that allows *H. pylori* to measure the concentration of the attractant and repellent substances, to flee undesirable conditions for growth. With chemotaxis, *H. pylori* can detect urea, metals and amino acids in the environment.⁵⁷⁻⁵⁹ It can detect injured epithelial cells that supply high concentrations of nutrients in the environment.⁶⁰ The antrum and the corpus are populated with differentiated cells, with variation in concentrations of nutrients and metabolites such as sugars and amino acids. *H. pylori*'s chemoreceptors, known as transducer like proteins, TlpA, TlpB, TlpC and TlpD, play an important role of inputting environment signals that the bacterium utilizes to determine the swimming direction toward a favorable environment to grow and colonize.⁶¹ For instance, *H. pylori*'s TlpA senses its attractant, arginine, and in response the bacterium swims toward the source of this essential amino acid.⁶² TlpB senses a repellent, auto-inducer 2 (AI-2), that allows *H. pylori* to determine the density of other bacteria in the surrounding area as it grows and colonizes in the stomach.⁶³

H. pylori produces two main cytotoxins: the cytotoxic associated gene (CagA) and the vacuolating cytotoxin (VacA).^{64,65} *H. pylori* adheres to the epithelial cells using adhesion factors to deliver CagA.^{66,67} Type IV secretion system (T4SS) delivers CagA upon attachment to the epithelial cells. Inside the human cells, CagA triggers the alteration of transduction signals and gene expression.⁶⁸⁻⁷⁰ As a result, different signaling pathways are affected, including pathways that participate in cell-cell tight junction and pro-inflammatory response. Upon interaction with the host cell, T4SS alone can trigger pro-inflammatory response and the production of the chemokine IL-8.⁷¹ Unlike CagA, *H. pylori* actively secretes VacA not requiring adhesion which interacts with the plasma membrane of host cells, leading to the formation of anion channels, Cl⁻ ions influx and the formation of vacuoles.⁷² Inside the host cell, VacA leads to the release of pro-inflammatory proteins and the induction of apoptosis.^{73,74} *H. pylori* benefits from

apoptosis of the host cells, which results in an increase of nutrients in the surrounding environment or diminished immunity that comes with the degradation of immune cells.⁷⁵

The Status of Discovery of the Anti-*H. pylori* Drugs

***H. pylori* Genome and Target-based Drug Discovery**

Advances in whole genome sequencing have provided opportunities for target-based drug discovery against *H. pylori*. Targeting the genes that promotes the growth and colonization of *H. pylori* is a promising strategy in the discovery of new therapies against *H. pylori* infections. In 1997, the complete genome sequence of the *H. pylori* 26695 strain became available.⁷⁷ A couple of years later, the genome of *H. pylori* J99 strain was reported.⁷⁸ The analysis of these genomes revealed that 60% of the genes produce proteins of known function based on the sequence similarity to their orthologs that are found in the public database.⁷⁹ 23% of the remaining genes have their corresponding orthologs in the public domain, however the function of their gene products is unknown. The rest of the genes (17%) have not been matched in the public database and the function of these genes have yet to be determined.

A recent transposon mutagenesis study identified 344 *H. pylori* essential genes.⁸⁰ The function of 24% of these *H. pylori* specific genes remains unknown. Some of these unique *H. pylori* genes are believed to enable *H. pylori*, to grow under the gastric mucosa, in close association with the epithelial cells.⁸¹ Other studies have discovered genes responsible for the motility of *H. pylori*, a function that facilitates *H. pylori* to penetrate and colonize in the mucus.⁸² Other genes have been associated with chemotaxis, a mechanism that enables *H. pylori* to survive the gastric acidity.^{83,84} More genes have identified by the knock out mutagenesis to be involved in different cellular functions such as cell envelope synthesis, cell division, protein synthesis, gene expression and regulation, cell metabolism and energetics.⁷⁹ The existence of these *H. pylori* specific genes suggest that *H. pylori* possesses unique proteins that can be targeted for the treatment of *H. pylori*.

Phenotypic/Highthrough-put Screening for Anti-*H. pylori* Drugs

The existence of *many* unique genes provides opportunities to selectively target *H. pylori*. The highthrough-put screen of compound library is a promising approach to identify active compounds against *H. pylori*. In addition, there is a high likelihood of finding selective hits against *H. pylori* by screening commercial libraries, given these libraries are often screened to identify broad-spectrum, while narrow-spectrum compounds are overlooked.

Examples of Novel Anti-*H. pylori* Compounds

Various research groups have published compounds that have demonstrated activity and selectivity against *H. pylori*. Katsura et al. published a series of guanidino analogs that demonstrates selectivity comparable to the standard of care drugs such as metronidazole and amoxicillin.⁸⁵⁻⁸⁹ The structure activity relationship of the guanidino compounds focused on the guanidine substitution and the 4 position of the thiazole ring.^{86,88-90} Among these guanidino analogs, compound 6 (**Figure 1-2A**) demonstrates the highest potency with an MIC of 0.17 $\mu\text{g/mL}$.⁸⁵ Ando et. al reported a series of arylacetamides analogs that were discovered through compound screening efforts.⁹¹ The lead compound, structure shown in **Figure 1-2G**, demonstrates high potency (MIC = 0.39 $\mu\text{g/mL}$), selectivity and stability in acidic conditions. The pyrazole derivatives have demonstrated activity and selectivity against *H. pylori* (**Figure 1-2H**) demonstrates good potency (MIC = 0.125 $\mu\text{g/mL}$) and selectivity against *H. pylori*.⁹²⁻⁹⁴ Dihydroorotate dehydrogenase (DHOase) was reported as the target of the pyrazole lead compound (MIC = 0.125 $\mu\text{g/mL}$, **Figure 1-2H**), a key enzyme in the *de novo* biosynthesis of pyrimidines. Other natural product compounds have demonstrated activity and selectivity against *H. pylori*, including the pyloricidins (A, B and C), the phthalide derivatives, the pyrone derivatives, Indolmycin and Calvatic acid.⁹⁵⁻¹⁰⁴ These compounds, structures shown in **Figure 1-2B, C, D, E and F**, have demonstrated potency with a MIC equivalent to 0.006 $\mu\text{g/mL}$, 5ng/mL and 0.003 $\mu\text{g/mL}$, 0.016 $\mu\text{g/mL}$ and 0.016 $\mu\text{g/mL}$ respectively.

The Benzimidazole Series

Researchers at AstraZeneca discovered a series of benzimidazole analogs including lead compound in **Figure 1-2I** with a high potency (MICs <0.5 $\mu\text{g/mL}$) and selectivity against diverse *H. pylori* strains.¹⁰⁵ In this study, resistant *H. pylori* mutants were generated with the lead compound of the series (**Figure 1-2I**).¹⁰⁶ The genomic DNA of the mutants were isolated and transformed into a sensitive strain (AH244), confirming that these DNAs can confer resistance. The genomic library of the mutant was constructed and mapped onto the *H. pylori* strain J99 genome to identify resistant genes. This study revealed a single point mutation in nuoD (NADH:Quinone oxidoreductase) that yielded the amino acid change G398S. NuoD was confirmed as the putative target, with the additional 32 independent spontaneous mutants that revealed missense mutations which resulted in the amino acid changes: G398S, F404S and V407M. NuoD is one of the polypeptides that make up *H. pylori*'s respiratory Complex I, a proton-pumping oxidoreductase that is located in the plasma membrane.¹⁰⁷ It catalyzes the first step of oxidative phosphorylation, by transferring 2 electrons from NADH to quinone. According to this study, the benzimidazoles bind at the interface of NuoD and the neighboring subunit NuoB. *H. pylori*'s complex I and *E. coli*'s complex I were sensitive to rotenone, an inhibitor of *Rhodobacter capsulatus*' complex I inhibitor, whereas the *E. coli*'s complex I was not sensitive to the benzimidazole lead (**Figure 1-2I**), suggesting that the benzimidazoles are selective against *H. pylori*.¹⁰⁸ Knockout mutagenesis experiments determined that nuoD was essential for the viability of *H. pylori* strain J99

Figure 1-2. Structures of published anti-*H. pylori* compounds.

(A) Guanidinothiazole derivative (MIC = 0.17 $\mu\text{g/mL}$). (B) Pyloricidin derivative (MIC < 0.006 $\mu\text{g/mL}$). (C) Phthalide derivative (MIC = 5.00 ng/mL). (D) Pyrone derivative (MIC = 0.003 $\mu\text{g/mL}$). (E) Indolmycin (MIC = 0.016 $\mu\text{g/mL}$). (F) Calvatic acid (MIC = 0.016 $\mu\text{g/mL}$). (G) Arylacetamide derivative (MIC = 0.39 $\mu\text{g/mL}$). (H) Pyrazole derivative (MIC = 0.125 $\mu\text{g/mL}$). (I) Benzimidazole derivative (MIC < 0.5 $\mu\text{g/mL}$). (J) HPi1 (MIC < 0.16 $\mu\text{g/mL}$).

and AH244. In *E. coli*, *nuoD* and *nuoC* genes are found fused and they encode a single polypeptide.¹⁰⁹ In *H. pylori*, these genes are separated, emphasizing the essentiality of *nuoD* in *H. pylori*, and not for other bacteria including *E. coli*.

The Discovery of Hpi1

Recently, we conducted a highthrough-put screen of 30,000 compounds to identify selective compounds with activity against *H. pylori*.⁸¹ The conditions for growing *H. pylori* were optimized in a 384 well plate, a challenging feat given the requirement of *H. pylori* for microaerophilic growth conditions. Promiscuous compounds were identified and eliminated studying the activity against a panel of commensal bacteria.

Bacterial growth was measured by reading the reduction of resazurin (blue dye), a metabolic indicator, to resorufin (pink fluorescent compound). The screen yielded HPi1 (**Figure 1-2J**), a selective hit compound, which demonstrates a high *in vitro* potency (IC₅₀ of 0.24 μ M) compared to Clarithromycin (0.04 μ M) and good selectivity against a panel of commensal bacteria such as *Lactobacillus casei*, *Lactobacillus reuteri* and *Bifidobacterium longum*. In the *in vivo* studies, HPi1 showed efficacy when tested in the *H. pylori* murine model at 25 mg/kg (p.o.). HPi1 reduces the colony counts to below the limit of detection. Attempts to generate resistant mutants against HPi1, by serial passage on agar plate or from HPi1 treated mice, were not successful and its target is unknown.⁵³

Repurposing Other Antibiotics for the Treatment of *H. pylori*

Fluoroquinolone compounds have been explored for the eradication of *H. pylori*. Finafloxacin, a novel fluoroquinolone, demonstrates superior anti-*H. pylori* activity in acidic conditions than other fluoroquinolones.¹¹⁰ Sitaflaxacin, a fluoroquinolone antibiotic, show higher anti-*H. pylori* activity (MIC₉₀: 0.06 μ g/mL) than metronidazole, clarithromycin, amoxicillin, tetracycline, doxycycline, minocycline, norfloxacin, ciprofloxacin and levofloxacin against 293 *H. pylori* strains, with low resistance development.¹¹¹ In combination with lansoprazole, sitafloxacin eradicates *H. pylori in vivo* at 83.3% rate. At 1 mg/kg, the C_{max} of sitafloxacin is 10 fold higher than its MIC. DX-619, a des-F(6)-quinolone, is active against gram-positive bacteria including MRSA and vancomycin resistant enterococci.¹¹² DX-619 also demonstrates bactericidal activity against 293 *H. pylori* strains, including those that are resistant to metronidazole, clarithromycin, amoxicillin and levofloxacin, with MIC₅₀ of 0.008 mg/L and MIC₉₀ of 0.06 mg/L.

Some macrolides have been reported to show anti-*H. pylori* activity. Azithromycin, a macrolide antibiotic, demonstrates an *in vitro* MIC₉₀ of 0.25 mg/L.¹¹³ Azithromycin is stable in acidic conditions and exhibits physical chemical properties that allows a rapid migration of drug from blood to tissue and a slow release from tissue to blood circulation. The evaluation of *in vivo* pharmacokinetic properties revealed that the concentration of Azithromycin in mucosa was significantly higher than in plasma,

ranging between 18.5 mg/kg and 24.6 mg/kg after a single oral administration (2.3-4.6 mg/kg). After 30 days of treatment with azithromycin at 500 mg/kg (+ omeprazole at 40 mg/kg), eradication of *H. pylori* was achieved in 80% of patients. Dirithromycin and roxithromycin demonstrates anti-*H. pylori* activity with MIC₅₀ of 0.25 and <0.008 µg/mL, respectively.¹¹⁴ These macrolides show acid stability unlike other macrolides such as erythromycin, ethylsuccinate, josamycin and spiramycin.

Marketed drugs to treat infections caused by parasites, have also investigated for growth inhibition of *H. pylori*. Artemisinin and its derivatives have been explored for their anti-*H. pylori* activity.¹¹⁵ Five artemisinin derivatives, b-artecyclopropylmethers, b-artether, a-artether, b-artemether and b-artefurfurylether, show anti-*H. pylori* activity and lack activity against the majority of other bacterial and fungal strains. B-artecyclopropylmethers is the most active with MIC of 0.25-1 µg/mL at the stomach pH. B-artecyclopropylmethers demonstrates mild *in vivo* efficacy at 50 mg/kg/day as a single therapy. The mechanism of action of the artemisinin derivatives is not well understood. Nitazoxanide (NTZ), a thiazolide drug, is a broad-spectrum drug used for the treatment of infections caused by parasites and helminths.¹¹⁶ NTZ exhibits activity against *H. pylori* and is a non-competitive inhibitor of pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR) of *H. pylori*. PFOR catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA and CO₂. It is only found in anaerobic bacteria, anaerobic parasites and epsilon proteobacteria.

Drugs targeting proton pump enzymes have demonstrated anti-*H. pylori* activity. Rabeprazole and its thioether derivative inhibits the motility of *H. pylori* with inhibition of 50% of motility at 16 and 0.25 µg/mL respectively.¹¹⁷ The inhibition of motility is observed instantly when bacteria is exposed to the agent, but growth inhibition is realized after hours of incubation. Lansoprazole, a benzimidazole proton pump inhibitor, show high activity against *H. pylori* with MIC of 3.13-12.5 µg/mL.¹¹⁸ Under acidic conditions, lansoprazole is transformed into AG-2000 and AG-1812, which show comparable activity to lansoprazole. These compounds lead to morphological change of cell, their constriction, collapse of cell surface structures and eventually cell death. Lansoprazole and its metabolites (AG-2000 and AG-1812) do not show activity at 100 µg/mL against aerobic, anaerobic bacteria and campylobacter jejuni. Lansoprazole is found highly distributed in the gastric mucosa and less in the serum. Idebenone and other quinones including duroquinone, menadione, juglone and coenzyme q1 inhibit *H. pylori* growth at the MIC₉₀ of 1.6-3.2 µg/mL.¹¹⁹ Idebenone inhibits respiration and triggers the reduction of the cellular ATP level, without showing adverse effect to human cells. Idebenone is selective against *H. pylori* and does not show activity against a variety of bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus salivarius* and *Pseudomonas aeruginosa*.

Other antibiotics and non-antibiotics have shown anti-*H. pylori* activity. B-lactams including cefuroxime, cefetamet, ceftetrame, cefixime and tigemonam, demonstrate activity against *H. pylori* with MIC₅₀ of 0.125, 2, 0.5, 0.06 and 0.25 µg/mL, respectively.¹¹⁴ The antimycotic ketoconazole is active against forty *H. pylori* strains at MIC₅₀ and MIC₉₀ of 16 and 32 mg/L respectively.¹²⁰ Mupirocin, a topical antimicrobial

for the treatment of staphylococcal infection of the skin, show activity against 57 strains of *H. pylori* with MIC₉₀ of 0.25 and 0.12 mg/L at pH 7.4 and 5.4 respectively.¹²¹ Linezolid, an oxazolidone indicated for staphylococcal and streptococcal infections, has demonstrated activity against gram-negative bacteria.¹²² Linezolid inhibits 70 strains of *H. pylori* at the MIC₅₀ of 8-64 µg/mL. Simethicone, a non-antibiotic and anti-foaming agent used to treat meteorism, demonstrates anti-*H. pylori* activity with an MIC of 64-128 mg/L.¹²³ HSR-903, a quinolone compound, demonstrates anti-*H. pylori* activity with MIC₅₀ of 0.20 µg/mL and MIC₉₀ of 12.5 µg/mL.¹²⁴

Research Goals

In conclusion, new therapies for the treatment of *H. pylori* infections are needed, given the rise of resistance against current antibiotics. The development of narrow spectrum anti-*H. pylori* drugs should be prioritized to reduce side effects from disruption of the microbiome. Advances in whole genome sequencing have revealed many *H. pylori* targets for the discovery of selective anti-*H. pylori* compounds. The highthrough-put screen of commercial libraries is an attractive approach in the discovery of selective compounds against *H. pylori*. However, lead optimization campaign of compounds discovered from a phenotypic screen is more challenging and takes a long time compared to compounds that are developed through target-based drug discovery approach. The generation of mutants and genome sequencing techniques enable the study of mode of action of novel antimicrobials. To eradicate *H. pylori* in vivo, compounds must demonstrate the ability to reach the colonization site, in the mucus layer of the stomach. The goals of the research presented in this dissertation are to identify active compounds against *H. pylori* through the highthrough-put screening approach, to explore the hit compounds through the structure-activity relationship for improved potency and to optimize the *in vitro* and *in vivo* pharmacokinetic properties the resulting lead compounds for proof of concept studies in *H. pylori* infected mice.

CHAPTER 2. THE DISCOVERY AND DEVELOPMENT OF THIENOPYRIMIDINES AS INHIBITORS OF HELICOBACTER PYLORI THROUGH INHIBITION OF THE RESPIRATORY COMPLEX I

Introduction

Helicobacter pylori is a major causative agent of gastritis, peptic ulcers and ultimately gastric cancer. According to the Center for Disease Control, more than 500,000 cases of *H. pylori* infections are diagnosed annually in the United States.¹ The triple therapy, consisting of a proton pump inhibitor and broad spectrum antibiotics clarithromycin and amoxicillin, is the recommended treatment against *H. pylori* infections.² The rate of successful treatment with the triple therapy has dropped below 80% due to the rise of resistance.³⁻⁶ In a recent study, 22% of patients tested in a clinical trial were not cured by the triple therapy.⁵ Additionally, the intensive use of the broad-spectrum antibiotics for *H. pylori* infections perturbs the gut microbiome, leading to side effects such as diarrhea and drug resistance in commensal pathogens.² Given the rise of *H. pylori* resistance, new drugs and approaches are needed for the eradication of *H. pylori* infections.

A high-throughput screening protocol was developed that addressed the *H. pylori* challenge of growing *H. pylori* in a fastidious microaerophilic environment required for growth, to discover novel chemical matter active against *H. pylori*.⁷ Hits were then counter-screened against *Staphylococcus aureus* to remove promiscuous compounds, against gut commensal bacteria and mammalian cell lines to discover selective hits that can eradicate *H. pylori* while preserving the host microbiota.⁷ From this approach we have previously reported Hpi1 (**Figure 2-1A**), a selective anti-*H. pylori* compound that emerged as a hit, with excellent *in vitro* potency (*H. pylori* IC₅₀ of 0.24 ± 0.04 μM) compared to clarithromycin (0.04 ± 0.01 μM) and amoxicillin (0.08 ± 0.04 μM).⁷ The screening approach was subsequently applied to a larger library of 219,197 compounds.⁸ 2,027 compounds emerged as positive hits and these compounds were clustered and subsequently prioritized based on their solubility and their chemical tractability. To eliminate generally toxic compounds, 511 were picked and counter-screened against *S. aureus* and gut commensals including *Bifidocacterium longum* ATCC BAA-999, *Lactobacillus casei* ATCC 334 and *Lactobacillus reuteri* ATXX 23272 and FaDu human cell line to eliminate promiscuous compounds. This screen identified two thienopyrimidine compounds, whose activity was confirmed by resynthesis and testing, **1** and **2** (**Figure 2-1A**), with the *H. pylori* IC₅₀ values of 0.46 μg/ml and 0.59 μg/ml respectively as prospective series leads.

Herein, we report the structure-activity relationship developed from the optimization thienopyrimidines hits **1** and **2** against *H. pylori*, efforts that examine substitutions around the thienopyrimidine core and merge elements of the two hits. Leads are developed based on their activity and cytotoxicity profiles, and their efficacy in the *in vivo* and *ex vivo* *H. pylori* infection models in mice is examined. Through resistance generation, whole genome sequencing and resistance transfer, NuoD subunit of

Figure 2-1. Structures of hit compounds from a highthrough-put screen against *H. pylori* and SAR development of the thienopyrimidines series.

(A) Structure of Hpi1, a selective anti-*H. pylori* compound that emerged as a positive hit from a high through-put screen against *H. pylori*. (B) and (C) Structures of thienopyrimidines hits **1** and **2**, that were identified using a similar screening approach with the *H. pylori* IC₅₀ of 0.46 µg/mL and 0.59 µg/mL respectively. (D) The Structure-Activity Relationship (SAR) of the thienopyrimidines against *H. pylori* is reported in four series, at the 4, 5 and 6 positions.

Complex I was discovered as the putative target and validated by resistance transfer. A homology model of the putative *H. pylori* NuoD binding site was created and the binding mode of the leads examined. These studies produced tractable thienopyrimidine leads with a defined mechanism of action suitable for further optimization of their ADME and *in vivo* properties.

Materials and Methods

Chemical Synthesis

Starting materials were purchased from the major vendors such as Sigma-Aldrich and Enamine. Chemical reactions were monitored by the thin-layer chromatography (TLC) and the Waters ACQUITY-UPLC-MS-UV system. Microwave-assisted chemical reactions were carried out in the Biotage Initiator⁺. The reaction mixtures were purified using the Biotage Flash column chromatography system, with silica cartridges acquired from Biotage Inc. The solvents for chromatography were purchased from Sigma-Aldrich. NMR spectra were obtained using the Bruker 400 MHz NMR spectrometer and the Bruker AVANCE 500 MHz NMR spectrometer. NMR analysis was carried out using the MestReNova software. The chemical shifts and the coupling constants (*J*) are reported in ppm and hertz (Hz) respectively. The purity of compounds was determined to be >95% by UPLC-MS-UV system and NMR. Optical rotation of the chiral compounds was determined using JASCO P-1010 Polarimeter.

Synthesis of Thienopyrimidine Analogs

Ethyl 2-amino-5-phenylthiophene-3-carboxylate (5). In a microwave tube, ethyl 3-cyanopropanoate **4** (1.771 mL, 16.65 mmol) was added to a solution of 2-phenylacetaldehyde **3** (2 g, 16.65 mmol), sulfur (0.587 g, 18.315 mmol) and morpholine (1.579 mL, 18.315 mmol) in ethanol (20 mL). The resulting mixture was submitted to microwave heating for 30 min at 70°C. After cooling, the mixture was filtered, and the filtrate was poured in water. The precipitate was collected, dried and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient 12-60% ethyl acetate in hexanes) to afford **5** as a yellow solid.

Ethyl 2-amino-5-phenylthiophene-3-carboxylate (5). ¹H NMR (DMSO, 500 MHz): δ 7.48-7.44 (m, 4H), 7.33 (t, *J* = 7.7 Hz, 2H), 7.23 (s, 1H), 7.19 (t, *J* = 7.4 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 1.28 (t, *J* = 7.1 Hz, 3H); MS (ESI): *m/z* 248.5 (M⁺+H).

6-phenylthieno[2,3-*d*]pyrimidin-4(3H)-one (6). Compound **5** (1.15 g, 4.65 mmol) was dissolved in excess formamide and the resulting mixture was heated for 16 h at 160°C. The mixture was concentrated in vacuo and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient 12-60% ethyl acetate in hexanes) to afford **6** as a red solid.

6-phenylthieno[2,3-d]pyrimidin-4(3H)-one (6). ¹H NMR (CDCl₃, 500 MHz): δ 11.05 (s, 1H), 8.58 (s, 1H), 7.65-7.60 (m, 2H), 7.45 (s, 1H), 7.43-7.38 (m, 2H), 7.33-7.30 (m, 1H); MS (ESI): *m/z* 230.1 (M⁺+H).

4-chloro-6-phenylthieno[2,3-d]pyrimidine (7). Compound **6** (1 g, 4.38 mmol) and N,N-dimethylaniline (0.043 mL, 0.336 mmol) were dissolved in phosphoryl trichloride (5.10 mL, 54.8 mmol) and the mixture was heated for 16 h to 95°C. The mixture was concentrated in vacuo and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient of 12-60% ethyl acetate in hexanes) to afford **7** as a yellow solid.

4-chloro-6-phenylthieno[2,3-d]pyrimidine (7). ¹H NMR (CDCl₃, 500 MHz): δ 8.85 (s, 1H), 7.79-7.77 (m, 2H), 7.62 (s, 1H), 7.54-7.46 (m, 3H); MS (ESI): *m/z* 247.1 (M⁺+H).

General procedure for the amination of 4-chloro-6-phenylthieno[2,3-d]pyrimidine. In a microwave tube, compound **7** (1 equiv), amine (1.5 equiv) and triethylamine (3 equiv) were dissolved in ethanol and subjected to microwave heating for 1 h at 150°C. The mixture was extracted with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, elution gradient of 20-85% ethyl acetate in hexanes) to afford the targeted compound.

3-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-1-ol (1). ¹H NMR (CDCl₃, 400 MHz): δ 8.48 (s, 1H), 7.69-7.67 (m, 1H), 7.67-7.66 (m, 1H), 7.48-7.43 (m, 2H), 7.41-7.37 (m, 1H), 7.33 (s, 1H), 5.70 (br, 1H), 3.85 (q, *J* = 6.5 Hz, 2H), 3.74 (t, *J* = 5.9 Hz, 2H), 1.93-1.87 (m, 2H); MS (ESI): *m/z* 286.1 (M⁺+H).

3-(methyl(6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-1-ol (8). ¹H NMR (CDCl₃, 400 MHz): δ 8.41 (s, 1H), 7.74 (s, 1H), 7.70-7.64 (m, 2H), 7.49-7.43 (m, 2H), 7.41-7.36 (m, 1H), 4.67 (br, 1H), 3.96 (dd, *J* = 6.8, 5.4 Hz, 2H), 3.61-3.60 (m, 2H), 3.51 (s, 3H), 1.96-1.90 (m, 2H); MS (ESI): *m/z* 301.1 (M⁺+H).

2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (9). ¹H NMR (CDCl₃, 400 MHz) δ 8.45 (d, *J* = 2.7 Hz, 1H), 7.72-7.60 (m, 2H), 7.43 (ddt, *J* = 9.5, 7.8, 1.5 Hz, 2H), 7.40-7.30 (m, 2H), 5.70 (s, 1H), 3.93 (dt, *J* = 5.0, 3.3 Hz, 2H), 3.82 (ddt, *J* = 7.2, 5.4, 3.0 Hz, 2H); MS (ESI): *m/z* 273.1 (M⁺+H).

2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-1-ol (10). ¹H NMR (CDCl₃, 400 MHz): δ 8.38 (s, 1H), 7.62-7.57 (m, 2H), 7.52 (d, *J* = 6.8 Hz, 3H), 7.37 (ddd, *J* = 7.7, 6.6, 1.6 Hz, 2H), 7.31-7.25 (m, 2H), 5.24 (d, *J* = 6.7 Hz, 1H), 4.42-4.40 (m, 1H), 3.81 (dd, *J* = 10.9, 3.1 Hz, 1H), 3.68-3.64 (m, 1H); MS (ESI): *m/z* 286.9 (M⁺+H).

1-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-2-ol (11). ¹H NMR (DMSO, 400 MHz): δ 8.33 (s, 1H), 8.12 (s, 1H), 7.99 (t, *J* = 5.9 Hz, 1H), 7.69 (d, *J* = 7.5

Hz, 2H), 7.51 (t, $J = 6.8$ Hz, 2H), 7.42-7.38 (m, $J = 7.5$ Hz, 1H), 4.86 (d, $J = 4.8$ Hz, 1H), 3.94-3.89 (m, 1H), 3.54-3.38 (m, 2H), 1.13 (d, $J = 6.2$ Hz, 3H); MS (ESI): m/z 268.7 ($M^+ + H$).

(S)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-1-ol (12). ^1H NMR (CDCl_3 , 400 MHz): δ 8.47 (s, 1H), 7.71-7.68 (m, 2H), 7.46 (t, $J = 7.5$ Hz, 2H), 7.41-7.39 (m, 1H), 7.35 (s, 1H), 5.31 (s, 1H), 4.51-4.49 (m, 1H), 3.91 (dd, $J = 11.1, 3.02$ Hz, 1H), 3.75 (dd, $J = 11.1, 6.2$ Hz, 1H), 1.41 (d, $J = 6.9$ Hz, 3H); MS (ESI): m/z 286.9 ($M^+ + H$).

(R)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)propan-1-ol (13). ^1H NMR (CDCl_3 , 400 MHz): δ 8.47 (s, 1H), 7.70-7.68 (m, 2H), 7.46 (t, $J = 8.2$ Hz, 2H), 7.41-7.35 (m, 2H), 5.33 (s, 1H), 4.52-4.48 (m, 1H), 3.93-3.88 (m, 1H), 3.78-3.73 (m, 1H), 1.41 (d, $J = 6.4$ Hz, 3H); MS (ESI): m/z 286.9 ($M^+ + H$).

N-(1-methoxypropan-2-yl)-6-phenylthieno[2,3-d]pyrimidin-4-amine (14). ^1H NMR (DMSO , 400 MHz): δ 8.34 (s, 1H), 8.12 (s, 1H), 7.74-7.68 (m, 3H), 7.51 (t, 2H, $J = 7.4$ Hz), 7.43-7.38 (m, 1H), 4.60-4.53 (m, 1H), 3.52-3.48 (m, 1H), 3.40-3.37 (m, 1H), 3.30 (s, 3H), 1.24 (d, $J = 6.7$ Hz, 3H); MS (ESI): m/z 300.8 ($M^+ + H$).

4-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)butan-1-ol (15). ^1H NMR (CDCl_3 , 400 MHz): δ 8.47 (s, 1H), 7.67-7.64 (m, 2H), 7.45-7.25 (m, 4H), 5.67 (s, 1H), 3.79 (s, 2H), 3.69 (s, 2H), 2.16 (s, 1H), 1.87-1.84 (m, 2H), 1.75-1.73 (m, 2H); MS (ESI): m/z 301.3 ($M^+ + H$).

2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)butan-1-ol (16). ^1H NMR (CDCl_3 , 400 MHz): δ 8.45 (s, 1H), 7.70-7.67 (m, 2H), 7.48-7.43 (m, 2H), 7.39 (t, $J = 7.48$ Hz, 1H), 7.35 (s, 1H), 5.34 (d, $J = 7.5$ Hz, 1H), 4.33-4.26 (m, 1H), 3.93 (dd, $J = 11.1, 3$ Hz, 1H), 3.80 (dd, $J = 11.1, 5.8$ Hz, 1H), 1.88-1.70 (m, 2H), 1.09 (t, $J = 7.2$ Hz, 3H); MS (ESI): m/z 300.9 ($M^+ + H$).

1-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)butan-2-ol (17). ^1H NMR (DMSO , 400 MHz): δ 8.33 (s, 1H), 8.12 (s, 1H), 7.96 (t, $J = 5.73$ Hz, 1H), 7.70-7.67 (m, 2H), 7.51 (dd, $J = 8.41, 7.01$ Hz, 2H), 7.42-7.38 (m, 1H), 4.83 (d, $J = 5.24$ Hz, 1H), 3.70-3.62 (m, 1H), 3.61-3.55 (m, 1H), 3.44-3.37 (m, 1H), 1.58-1.48 (m, 1H), 1.42-1.31 (m, 1H), 0.93 (t, $J = 7.4$ Hz, 3H); MS (ESI): m/z 300.9 ($M^+ + H$).

3-methyl-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)butan-1-ol (18). ^1H NMR (CDCl_3 , 400 MHz): δ 8.35 (s, 1H), 7.62-7.59 (m, 2H), 7.37 (t, $J = 7.2$ Hz, 2H), 7.32-7.25 (m, 2H), 5.30 (d, $J = 7.7$ Hz, 1H), 4.11-4.05 (m, 1H), 3.87-3.75 (m, 2H), 3.48 (m, 1H), 2.10-2.01 (m, 1H), 1.20-0.98 (m, 6H); MS (ESI): m/z 315.01 ($M^+ + H$).

N-(3,4-dimethoxyphenethyl)-6-phenylthieno[2,3-d]pyrimidin-4-amine (19). ^1H NMR (CDCl_3 , 400 MHz): δ 8.54 (s, 1H), 7.65 (d, $J = 7$ Hz, 2H), 7.45 (t, $J = 7.04$ Hz, 2H), 7.40-7.36 (m, 1H), 7.20 (s, 1H), 6.88-6.78 (m, 3H), 5.28 (br, 1H), 3.95-3.90 (m, 5H), 3.87 (s, 3H), 2.99 (t, $J = 7.04$ Hz, 2H); MS (ESI): m/z 392.01 ($M^+ + H$).

***N*-(3,4-dimethylphenethyl)-6-phenylthieno[2,3-*d*]pyrimidin-4-amine (20).** ¹H NMR (DMSO, 400 MHz): δ 8.37 (s, 1H), 8.07 (t, *J* = 5.62 Hz, 1H), 8.00 (s, 1H), 7.67 (d, *J* = 7.52 Hz, 2H), 7.51 (t, *J* = 8.08 Hz, 2H), 7.40 (t, *J* = 7.12 Hz, 1H), 7.06-7.05 (m, 2H), 6.99-6.97 (m, 1H), 3.73-3.68 (m, 2H), 2.87 (t, *J* = 7.49 Hz, 2H), 2.19 (s, 3H), 2.17 (s, 3H); MS (ESI): *m/z* 361.21 (M⁺+H).

6-phenyl-*N*-(2-(pyridin-4-yl)ethyl)thieno[2,3-*d*]pyrimidin-4-amine (21). ¹H NMR (CDCl₃, 400 MHz): δ 8.56-8.47 (m, 3H), 7.65-7.63 (m, 2H), 7.44-7.41 (m, 2H), 7.38-7.36 (m, 1H), 7.21-7.18 (m, 3H), 5.26 (s, 1H), 3.98-3.93 (m, 2H), 3.05 (t, 2H, *J* = 7.52 Hz); MS (ESI): *m/z* 333.1 (M⁺+H).

6-phenyl-*N*-(2-(pyridin-3-yl)ethyl)thieno[2,3-*d*]pyrimidin-4-amine (22). ¹H NMR (CDCl₃, 400 MHz): δ 8.56-8.53 (m, 3H), 7.68-7.65 (m, *J* = 6.88 Hz, 2H), 7.61 (d, *J* = 7.91 Hz, 1H), 7.45 (dd, *J* = 8.45, 6.60 Hz, 2H), 7.40-7.37 (m, 1H), 7.24 (s, 1H), 5.26 (s, 1H), 3.96 (q, *J* = 6.73, 2H), 3.08 (t, *J* = 6.96 Hz, 2H); MS (ESI): *m/z* 333.1 (M⁺+H).

***N*-(2,3-dimethoxybenzyl)-6-phenylthieno[2,3-*d*]pyrimidin-4-amine (23).** ¹H NMR (CDCl₃, 400 MHz) δ 8.54 (s, 1H), 7.69-7.63 (m, 2H), 7.49-7.41 (m, 2H), 7.40-7.33 (m, 1H), 7.30 (d, *J* = 1.2 Hz, 1H), 7.11-7.00 (m, 2H), 6.94 (dd, *J* = 7.8, 1.8 Hz, 1H), 5.73 (s, 1H), 4.89 (d, *J* = 5.6 Hz, 2H), 3.97 (d, *J* = 0.9 Hz, 3H), 3.92 (d, *J* = 1.1 Hz, 3H); MS (ESI): *m/z* 379.02 (M⁺+H).

3-(4-methoxyphenyl)-2-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)propan-1-ol (24). ¹H NMR (CDCl₃, 500 MHz): δ 8.38 (s, 1H), 7.55 (d, *J* = 7.65 Hz, 2H), 7.36 (t, *J* = 7.54 Hz, 2H), 7.30 (t, *J* = 7.7 Hz, 1H), 7.14-7.11 (m, 3H), 6.80 (d, *J* = 8.2 Hz, 2H), 5.39 (d, *J* = 6.25 Hz, 1H), 4.45-4.39 (m, 1H), 3.82 (d, *J* = 10.8 Hz, 1H), 3.72-3.66 (m, 4H), 2.97-2.80 (m, 2H); MS (ESI): *m/z* 391.9 (M⁺+H).

2-(4-methoxyphenyl)-2-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)ethan-1-ol (25). ¹H NMR (CDCl₃, 400 MHz): δ 8.38 (s, 1H), 7.59 (dd, *J* = 8.19, 1.45 Hz, 2H), 7.36 (td, *J* = 7.63, 6.91, 1.15 Hz, 2H), 7.32-7.27 (m, 4H), 6.86 (d, *J* = 8.48 Hz, 2H), 5.65 (d, *J* = 6.22 Hz, 1H), 5.35-5.31 (m, 1H), 4.01-3.98 (m, 2H), 3.75 (s, 3H), 3.57 (s, 1H); MS (ESI): *m/z* 378.1 (M⁺+H).

(*R*)-2-(4-methoxyphenyl)-2-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)ethan-1-ol (26). ¹H NMR (DMSO, 400 MHz): δ 8.28 (s, 1H), 8.24 (s, 1H), 8.13 (d, *J* = 8.2 Hz, 1H), 7.73-7.70 (m, 2H), 7.54-7.50 (m, *J* = 8.12 Hz, 2H), 7.43-7.39 (m, 1H), 7.37-7.34 (m, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 5.43-5.38 (m, 1H), 4.99 (t, *J* = 6.16 Hz, 1H), 3.79-3.69 (m, 5H); MS (ESI): *m/z* 378.1 (M⁺+H).

(*S*)-2-(4-methoxyphenyl)-2-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)ethan-1-ol (27). ¹H NMR (DMSO, 400 MHz): δ 8.28 (s, 1H), 8.24 (s, 1H), 8.14 (d, *J* = 7.76 Hz, 1H), 7.72 (d, *J* = 8.2 Hz, 2H), 7.52 (t, *J* = 7.72 Hz, 2H), 7.43-7.35 (m, 3H), 6.89 (d, *J* = 9 Hz, 2H), 5.41 (q, *J* = 7.88 Hz, 1H), 4.99 (t, *J* = 6.04 Hz, 1H), 3.79-3.69 (m, 5H); MS (ESI): *m/z* 378.1 (M⁺+H).

(R)-2-phenyl-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (28). ¹H NMR (CDCl₃, 400 MHz): δ 8.46 (s, 1H), 7.69 (d, *J* = 6.88 Hz, 2H), 7.47-7.34 (m, 9H), 5.85 (s, 1H), 5.50-5.47 (m, 1H), 4.17-4.09 (m, 2H); MS (ESI): *m/z* 348.9 (M⁺+H).

(R)-2-(2-fluoro-4-methoxyphenyl)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (29). ¹H NMR (CDCl₃, 400 MHz): δ 8.45 (s, 1H), 7.67 (d, *J* = 6.16 Hz, 2H), 7.45-7.29 (m, 5H), 6.70 (s, 2H), 6.02 (s, 1H), 5.70 (s, 1H), 4.14-4.05 (m, 2H), 3.81 (s, 3H); MS (ESI): *m/z* 396.01 (M⁺+H).

(R)-2-(2-fluoro-4-methoxyphenyl)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (30). ¹H NMR (CDCl₃, 400 MHz): δ 8.42 (s, 1H), 7.67-7.65 (m, 2H), 7.54 (s, 1H), 7.45-7.35 (m, 3H), 7.22-7.16 (m, 2H), 6.98 (t, *J* = 8.6 Hz, 1H), 6.31 (s, 1H), 5.45-5.41 (m, 1H), 4.14-4.06 (m, 2H), 3.90 (s, 3H); MS (ESI): *m/z* 397.3 (M⁺+H).

Compounds **37-40** were synthesized following the General procedure for the amination of 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

(R)-2-((5,6-dimethylthieno[2,3-d]pyrimidin-4-yl)amino)-2-phenylethan-1-ol (37). ¹H NMR (CDCl₃, 400 MHz): δ 8.35 (s, 1H), 7.44-7.28 (m, 5H), 6.28 (s, 1H), 5.47-5.43 (m, 1H), 4.07 (d, *J* = 4.58 Hz, 2H), 2.51 (s, 3H), 2.46 (s, 3H); MS (ESI): *m/z* 301.00 (M⁺+H).

(R)-2-((5-methyl-6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)-2-phenylethan-1-ol (38). ¹H NMR (CDCl₃, 400 MHz): δ 8.48 (s, 1H), 7.48-7.36 (m, 9H), 7.11-7.07 (m, 1H), 5.89 (d, *J* = 6.84 Hz, 1H), 5.49 (q, *J* = 5.44 Hz, 1H), 4.17-4.09 (m, 2H), 1.28 (s, 3H); MS (ESI): *m/z* 361.9 (M⁺+H).

(R)-2-((6-methyl-5-phenylthieno[2,3-d]pyrimidin-4-yl)amino)-2-phenylethan-1-ol (39). ¹H NMR (CDCl₃, 400 MHz): δ 8.42 (s, 1H), 7.57 (t, *J* = 7.63 Hz, 1H), 7.45-7.41 (m, 2H), 7.27-7.21 (m, 5H), 6.90-6.88 (m, 2H), 5.33 (d, *J* = 5.6 Hz, 1H), 5.20-5.16 (m, 1H), 3.78-3.69 (m, 2H), 2.33 (s, 3H); MS (ESI): *m/z* 361.9 (M⁺+H).

(R)-2-phenyl-2-((5-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (40). ¹H NMR (CDCl₃, 400 MHz): δ 8.45 (s, 1H), 7.69-7.67 (m, 2H), 7.47-7.35 (m, 9H), 5.96-5.94 (m, 1H), 5.51-5.47 (m, 1H), 4.17-4.10 (m, 2H); MS (ESI): *m/z* 348.1 (M⁺+H).

Synthesis of Amine Analog 31

tert-butyl (R)-(1-(3-fluoro-4-methoxyphenyl)-2-hydroxyethyl)carbamate (33). Di-*tert*-butyl dicarbonate (0.290 g, 1.329 mmol) was added to a solution of (R)-1-(3-fluoro-4-methoxyphenyl)-2-hydroxyethanaminium chloride **32** (0.267 g, 1.208 mmol) and triethylamine (0.506 mL, 3.63 mmol) in DCM (4 mL). The reaction mixture was stirred for 2 h at room temperature. The mixture was concentrated and purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-80% ethyl acetate in hexanes). Pure fractions were evaporated to afford **33** as a white solid.

tert-butyl (R)-(1-(3-fluoro-4-methoxyphenyl)-2-hydroxyethyl)carbamate (33). ¹H NMR (CDCl₃, 500 MHz): δ 7.08-7.03 (m, 2H), 6.96 (t, *J* = 8.33 Hz, 1H), 5.21 (d, *J* = 6.55 Hz, 1H), 4.73 (s, 1H), 3.90-3.81 (m, 5H), 2.11 (s, 1H), 1.46 (s, 9H); MS (ESI): *m/z* 284.3 (M⁺+H).

tert-butyl (R)-(2-(1,3-dioxisoindolin-2-yl)-1-(3-fluoro-4-methoxyphenyl)ethyl)carbamate (34).¹⁴⁴ DIAD (0.282 mL, 1.458 mmol) was added dropwise at room temperature, to a solution of **33** (0.320 g, 1.122 mmol), phthalimide (0.215 g, 1.458 mmol) and Ph₃P (0.382 g, 1.458 mmol) in THF (3.8 mL). The resulting mixture was stirred for 16 h at room temperature. The mixture was extracted with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-60% ethyl acetate in hexanes), to afford **34** as a yellow solid.

tert-butyl (R)-(2-(1,3-dioxisoindolin-2-yl)-1-(3-fluoro-4-methoxyphenyl)ethyl)carbamate (34). ¹H NMR (CDCl₃, 500 MHz): δ 7.80 (m, 2H), 7.66 (m, 2H), 7.04 (m, 2H), 6.87 (m, 1H), 5.28 (d, *J* = 8.1 Hz, 1H), 4.94-4.92 (m, 1H), 3.89-3.81 (m, 5H), 1.20 (s, 9H); MS (ESI): *m/z* 415.7 (M⁺+H).

(R)-2-(2-amino-2-(3-fluoro-4-methoxyphenyl)ethyl)isoindoline-1,3-dione (35) and (R)-2-(2-(3-fluoro-4-methoxyphenyl)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethyl)isoindoline-1,3-dione (36). HCl (4 M in 1,4-dioxane, 0.9 mL, 3.6 mmol) was added to a solution of **34** (0.372 g, 0.897 mmol) in a mixture of DCM (2.5 mL) and methanol (1 mL), and the resulting mixture was stirred for 16 h at room temperature. The mixture was subjected to acid-base extraction with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **35** (yellow oil) was used in the next step without further purification. **36** was synthesized following the general procedure for amination of 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

(R)-2-(2-amino-2-(3-fluoro-4-methoxyphenyl)ethyl)isoindoline-1,3-dione (35). ¹H NMR (MeOD, 500 MHz): δ 7.88-7.78 (m, 4H), 7.20 (dd, *J* = 12.4, 2.20 Hz, 1H), 7.08 (dd, *J* = 8.65, 2.14 Hz, 1H), 7.00 (t, *J* = 8.49 Hz, 1H), 4.25 (t, *J* = 7.27 Hz, 1H), 3.88-3.84 (m, 5H); MS (ESI): *m/z* 315.5 (M⁺+H).

(R)-2-(2-(3-fluoro-4-methoxyphenyl)-2-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)ethyl)isoindoline-1,3-dione (36). ¹H NMR (CDCl₃, 500 MHz): δ 8.31 (s, 1H), 7.88-7.85 (m, 2H), 7.75-7.73 (m, 4H), 7.54 (s, 1H), 7.49 (t, *J* = 7.55, 1H), 7.42 (t, *J* = 7.35 Hz, 1H), 7.25-7.22 (m, 2H), 6.99-6.95 (m, 1H), 6.91 (s, 1H), 5.66-5.62 (m, 1H), 4.24-4.12 (m, 2H), 3.89 (s, 3H); MS (ESI): *m/z* 525.5 (M⁺+H).

(R)-1-(3-fluoro-4-methoxyphenyl)-N¹-(6-phenylthieno[2,3-d]pyrimidin-4-yl)ethane-1,2-diamine (31). Compound **36** (0.203 g, 0.393 mmol) was dissolved in methanol and a 80 % hydrazine hydrate solution (0.073 mL, 1.2 mmol) was added dropwise to the solution at 0°C. The reaction mixture was stirred for 16 h at room

temperature. The mixture was extracted with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 2-20 % methanol in ethyl acetate) to afford **31** as a yellow solid.

(R)-1-(3-fluoro-4-methoxyphenyl)-N¹-(6-phenylthieno[2,3-d]pyrimidin-4-yl)ethane-1,2-diamine (31). ¹H NMR (DMSO, 500 MHz): δ 8.27 (s, 1H), 8.15 (s, 1H), 7.75 (d, 2H, *J* = 7.6 Hz), 7.54 (t, 2H, *J* = 7.6 Hz), 7.44 (t, 1H, *J* = 7.6 Hz), 7.26 (dd, *J* = 12.5, 2 Hz, 1H), 7.20 (d, 1H, *J* = 7.6 Hz), 7.12 (t, *J* = 8.6 Hz, 1H), 5.28-5.25 (m, 1H), 3.80 (s, 3H), 3.00-2.91 (m, 2H), 1.25-1.18 (m, 2H); MS (ESI): *m/z* 396.4 (M⁺+H).

***H. pylori* IC₅₀ Dose Responses**

H. pylori strains ATCC 43504, SS1, or SS1 NuoD A402P (see below) were grown overnight in brucella broth containing Skirrow's selective medium supplement ¹ and 10% FBS or brain heart infusion broth containing Skirrow's and 10% FBS. Cultures were grown in vented 25-cm² tissue culture flasks at 37°C, 10% CO₂ with rotation at 50 rpm. Two-fold serial dilutions of test and control compounds were performed in DMSO or water as appropriate and 1 uL was transferred to a 96 well plate. 99 uL of a 1:10 dilution of an overnight culture of *H. pylori* in Brucella/Skirrow's/10% FBS was added to each well. Plates were incubated for 18-20 h, and 5 uL of 3mM resazurin was added to each well. Plates were incubated for an additional 3-5 h, and resazurin reduction was measured using a BioTek Synergy Mx (BioTek, Winooski, VT) with an excitation wavelength of 540nm and emission read at 590nm. Percent inhibition compared to growth controls were calculated and the 50% inhibitory concentration (IC₅₀) was determined using XLFit software (IDBS, London, United Kingdom).

Cytotoxicity Assays

FaDu (ATCC HTB-43) and HepG2 (ATCC HB-8065) cell lines were routinely maintained according to the protocols provided by the American Type Culture Collection (ATCC). For cytotoxicity IC-50 determination, 100uL of cell suspension in Eagle's Minimal Essential Medium supplemented with 10% FBS was seeded into black sided, tissue culture treated 96 well plates (Corning 3603) at a concentration of 3 x 10⁵ cells/ml. Plates were incubated for 24 hours in a 37°C, 5% CO₂ incubator to allow for cell adhesion and growth. Spent media was aspirated from the test plates and serial two-fold dilutions of test compounds mixed with fresh EMEM + 10% FBS were added to the plates at 100 uL per well. The final DMSO concentration was 1%. The plates were incubated for 72 hours. The media was aspirated and FBS-free EMEM and resazurin (final concentration 0.15 mM) was added to the plate. Plates were incubated for 3-5 hours and then resazurin reduction was measured using a BioTek Synergy Mx (BioTek, Winooski, VT) with an excitation wavelength of 540nm and emission read at 590nm. Percent inhibition compared to growth controls were calculated and the 50% inhibitory

concentration (IC₅₀) curves were generated using XLFit software (IDBS, London, United Kingdom).

Generation of Resistant Mutants, Sequencing and Target Identification

Mutants resistant to the thienopyrimidine series were generated in two separate ways. Strain HM-274 was chosen for these studies, since it had been previously annotated. Spontaneous mutants of HM-274 were selected by concentrating and harvesting susceptible wildtype cultures and spreading them onto plates containing 2X MIC of **28**. Two stably resistant isolates were recovered which had MICs 8-fold higher than the wildtype. *H. pylori* ATCC 43504 was serially passaged onto plates containing increasingly higher concentrations of **1** until the strain was able to grow at 8X the MIC of the wild-type. The DNA from this resistant mutant was then purified and transformed into strain HM-274 and resistance was confirmed. Genomic DNA was purified from the three resistant mutants as well as HM-274 wildtype using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research) according to the manufacturer's instructions. Whole genome sequencing was performed by Tufts Medical Center. Genome annotation was accomplished using RAST and single nucleotide polymorphism (SNP) identification was performed with CLC Workbench. The two spontaneous resistant mutants had SNPs in *nuoD* which caused one amino acid change, either A402P or T400I. The serial passage mutant had multiple non-synonymous mutations in *nuoD*. There were no other shared mutations in all three strains.

Seven additional spontaneous resistant mutants were isolated. Genomic DNA was purified from each and *nuoD* was amplified by PCR. The PCR products were sequenced and compared to wildtype *nuoD*. All seven had mutations in *nuoD* resulting in an amino acid change, either A402P or T400I.

In order to ensure that no secondary mutations were causing thienopyrimidine resistance, a wild-type copy of *nuoD* was modified to contain the A402P mutation using the Q5 Site Directed Mutagenesis kit (New England Biolabs) according to the manufacturer's instructions. The mutation was confirmed by PCR and then wild-type SS1 was transformed with this plasmid and selected for on plates containing **28**. The A402P mutation in *nuoD* conferred resistance in strain SS1. Several colonies were chosen and the mutation was confirmed by PCR and sequencing. An SS1 isolate with the NuoD A402P mutation was used in further testing to confirm on target activity of the thienopyrimidine series (see above).

***ex vivo* Efficacy Study**

This model was modified from what has previously been described²⁻⁴. Briefly, 6- to 8-week-old female C57BL/6 (specific-pathogen-free) mice were obtained from Charles River Laboratories. Five mice were housed per cage and allowed to acclimate for

at least 72 h. Animals were inoculated by oral gavage, three times within one week with 0.2 ml of an *H. pylori* SS1 suspension ($\sim 10^9$ CFU/dose). The infection was allowed to stabilize for one to two weeks after the final gavage. Mice were euthanized and the stomachs were excised, bisected laterally into equal halves along the long curvature, and washed three times with sterile PBS. Each half was then added to the well of a 12 well plate containing Brucella broth supplemented with 10% FBS and Skirrow's with or without 100X the MIC of Compound **25**. Each half from individual stomachs were assigned to a control and treatment group. After 24 h, the stomach halves were washed, weighed, homogenized, serially diluted, and plated on selective medium. These plates were incubated for 7 days and then colonies were counted to determine colony counts. Data was log transformed and statistical significance was determined using a Mann-Whitney test with GraphPad Prism software. A P-value less than 0.05 was considered significant. The study was repeated to ensure reproducibility. Animal use complied with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

Homology Modeling of *H. pylori*'s Complex I

A homology model of the subunits NuoD and NuoB of NADH:ubiquinone Oxidoreductase in *Helicobacter pylori* was created using Schrodinger's Maestro program. The homology model was built using the protein sequence from a *Thermus thermophilus* crystal structure (PDB 2FUG) as a template to build the NuoD and NuoB subunits of NADH:quinone oxidoreductase in *H. pylori*. A series of thienopyrimines and two known complex I inhibitors were docked using Schrodinger's Induced Fit docking based upon the quinone binding channel, based on previous *Thermus thermophilus* and mitochondrial studies of ubiquinone binding (Tyr85, Arg40, Val403, and Asn401), and from a ligand in our thienopyrimidine series (T400 and A402).⁷ All residue numbers are based on the protein sequence of *H. pylori*. A multiple sequence alignment was performed with mouse, bovine, human, *A. fumigatis*, *H. pylori*, *E. coli* and *M. tuberculosis* using CLC Genomic Workbench 11.

Results and Discussion

New compounds were synthesized to develop the SAR around the two thienopyrimidine hits as outlined in **Scheme 2-1**.¹²⁵⁻¹²⁸ First, phenylacetaldehyde **3** is reacted with ethyl 3-cyanopropanoate **4** to give phenylthiophene **5**. Heating **5** in the excess of formamide formed pyrimidone **6**. Chlorination and dehydration of the pyrimidone with phosphoryl chloride afforded the chlorothienopyrimidine **7**, which was coupled with a variety of primary and a secondary amine to give targeted 4-position substituted *H. pylori* inhibitors (**8-30**). These amines were selected based on their structural resemblance to the amine substituents of the hit compounds and the progress of the anti-*H. pylori* SAR. Initial SAR efforts focused on exploring the 4-position of the thienopyrimidine core, examining the N-alkyl hydroxyl moiety of hit compound **1**, Series

Scheme 2-1. Synthesis of thienopyrimidine derivatives.

Reagents and conditions: (i) Sulfur, Morpholine, Ethanol, 70°C, Microwave, 20 min, 90%; (ii) Formamide, reflux, 18 h, 80%; (iii) POCl₃, N, N dimethylaniline, reflux, 14 h, 90%; (iv) Ethanol, 150°C, Microwave, 1 h, 80-93%.

1 (**Table 2-1**) and phenethyl amine motif of compound **2**, Series 2 (**Table 2-2**). These sidechains were then successfully merged into Series 3 (**Table 2-3**). Commercially available amine starting materials with hydroxyl and aromatic moieties, similar to the moieties identified with the hit compounds, were used to synthesize analogs in these series to investigate the SAR at the 4-position.

In series 1, thienopyrimidines variation to the propyl alcohol like substituent of hit compound **1** were examined (**Table 2-1**). Compound **8** was generated first to investigate the effect of N-methylation of the amine group attached at the 4-position of the thienopyrimidine found in hit compound **1**. Compound **8** demonstrates less anti-*H. pylori* activity and also increases the lipophilicity negatively impacting the Lipophilic Efficiency (LLE), suggesting that the free secondary amine is preferred in this series. To optimize the spacer between the hydroxyl group and the amine moiety, we synthesized compound **9** with an ethyl motif as spacer. Compared to hit compound **1**, **9** demonstrates less activity compared to compound **1**, suggesting that a propyl linker is preferred for improved potency. To investigate branching between the amine moiety at the 4-position and the hydroxyl group of compound **1**, we synthesized methyl branched isomers **10** and **11**. Compound **10** demonstrates higher potency and selectivity compared to compound **11**, suggesting further SAR development are preferred at the α -carbon to the amine over the β carbon. The methylation creates a chiral center, the contribution of each isomer in **10** was examined through the synthesis of enantiomers **12** (*S*) and **13** (*R*). The *R* enantiomer **13** demonstrates 75-fold increased activity over the *S* enantiomer **12**. To examine the contribution of the hydroxyl group, we synthesized compound **14**, the O-methylated analog of **10**. Compared to compound **10**, **14** demonstrates less potency with a large decrease in LLE, suggesting that the free hydroxyl group is desired for optimal activity. To expand on the linker region, we synthesized isomeric compounds **15**, **16** and **17** with an additional methylene unit. Compound **16**, α -carbon ethyl substituted analog, demonstrates the highest potency of these 3 isomers consistent with previously developed SAR. Compound **18**, with a bulkier isopropyl substitution over the ethyl motif found in **16**, showed much decreased potency. Overall, within this compound series clear SAR was observed consistent with the analogs engaging a defined molecular target.

Next compounds we examined how phenethyl sidechain of compound **2** performs on the 6-phenyl- thienopyrimidine core found in the first series, four analogs with varying phenyl substitutions were designed and synthesized **19**, **20**, **21** and **22** (**Table 2-2**). Best among these compounds, **19** displays the excellent potency and selectivity against *H. pylori*. **19** is the direct phenyl- thienopyrimidine hybrid of **2** with the 3,4-dimethoxyphenethyl side chain. Substitution of the dimethoxyphenyl motif with dimethylphenyl, compound **20**, decrease comparative potency and increased lipophilicity. 3 and 4 substituted pyridyl analogs (**21** and **22**) designed to increase metabolic stability and solubility also showed substantially decreased potency, suggesting the dimethoxyphenylethyl substitution provides optimum anti-*H. pylori* activity. To determine the optimum spacer for the phenyl moiety, we synthesized compound **23** with a methylene motif as spacer. **23** demonstrates less potency compared to **19**, suggesting the ethyl motif is preferred as spacer for this series of compounds.

Table 2-1. The structure-activity relationship of thienopyrimidines of series 1.

Compound		Log P	<i>H. pylori</i> IC ₅₀ ± SEM (µg/mL) SS1 strain ^a	Cytotoxicity IC ₅₀ ± SEM (µg/mL) FaDu Cells ^a	LLE ^b
1		3.36	0.46 ± 0.07	75.4 ± 42.6	-1.92
8		4.14	1.85 ± 1.48	29.55 ± 2.82	-1.16
9		3.25	0.546 ± 0.219	28.21 ± 5.36	-1.77
10		3.57	0.05 ± 0.09	26.74 ± 4.57	-2.68
11		3.57	0.13 ± 0.07	18.54 ± 0.97	-1.74
12		3.57	0.98 ± 0.71	79.44 ± 14.31	-1.57
13		3.57	0.01 ± 0.01	8.17 ± 1.10	-2.69
14		3.93	0.16 ± 0.09	10.13 ± 1.93	-2.38
15		3.81	0.18 ± 0.12	55.27 ± 1.59	-2.71
16		4.05	0.02 ± 0.00	11.66 ± 0.05	-3.24
17		4.05	0.09 ± 0.05	12.25 ± 0.05	-2.65
18		4.45	0.24 ± 0.05	15.64 ± 5.08	-3.37

^a All IC₅₀ data are the mean of 3 measurements. ^b LLE = pIC₅₀ – log P.

Table 2-2. The structure-activity relationship of thienopyrimidines of series 2.

Compound		Log P	<i>H. pylori</i> IC ₅₀ ± SEM (µg/mL) SS1 strain ^a	Cytotoxicity IC ₅₀ ± SEM (µg/mL) FaDu Cells ^a	LLE ^b
19		5.53	0.02 ± 0.002	133.34 ± 57.73	-4.69
20		6.75	0.22 ± 0.17	<6.25 ± 0.00	--5.83
21		4.44	0.48 ± 0.32	29.32 ± 4.81	-3.32
22		4.44	1.08 ± 0.47	30.31 ± 0.64	-2.72
23		5.25	0.237 ± 0.167	9.72 ± 3.00	-3.91

^a All IC₅₀ data are the mean of 3 measurements. ^b LLE = pIC₅₀ – log P.

Table 2-3. The structure-activity relationship of thienopyrimidines of series 3.

Compound		Log P	<i>H. pylori</i> IC ₅₀ ± SEM (μg/mL) SS1 strain ^a	Cytotoxicity IC ₅₀ ± SEM (μg/mL) FaDu Cells ^a	LLE ^b
24		5.12	0.09 ± 0.03	6.51 ± 0.14	-4.07
25		4.84	0.006 ± 0.003	161.58 ± 33.42	-3.93
26		4.84	0.004 ± 0.000	6.4 ± 0.01	-3.94
27		4.84	0.05 ± 0.04	21.44 ± 4.19	-3.86
28		4.96	0.01 ± 0.007	<6.25 ± 0.00	-4.07
29		4.99	0.004 ± 0.001	13.21 ± 0.60	-4.08
30		4.99	0.004 ± 0.000	12.42 ± 0.82	-4.08
31		4.61	0.54 ± 0.36	19.06 ± 7.40	-2.69

^a All IC₅₀ data are the mean of 3 measurements. ^b LLE = pIC₅₀ – log P.

The SAR progression revealed series leads **16** and **19** from hits **1** and **2** respectively. We hypothesized that merging both substituents at the 4-position of phenylthienopyrimidine could produce analogs with increased potency. Thus, we synthesized compound **24** (Table 2-3) which demonstrated higher activity than **16** and similar potent activity to **19**. In attempt to optimize the activity of **24** we were able to purchase several 2-phenylglycinol building block with a shorter spacer between the aryl motif and the nitrogen. First compound **25** was generated which demonstrates the highest potency of the series, in low ng/mL-range of activity (H.p. IC₅₀ = 0.006 µg/ml). Both enantiomers of **25** were then synthesized. Compound **26**, the R enantiomer of **25**, showed higher potency (H.p. IC₅₀ = 0.004 µg/ml) than the S enantiomer (**27**). To further understand the SAR around the phenylglycinol aryl ring, we synthesized fluorinated compounds, **28** and **29**. These compounds demonstrated comparable potency to **26**.

While various aryl rings at the 4-position of compound **28-30** demonstrate activity, we synthesized primary amine analog **31** to further elucidate the importance of the hydroxyl moiety and the SAR at the 4-position, as primary amines have been shown to enhance uptake of drugs into Gram-negative bacteria.¹²⁹ Compound **31** was synthesized as described in Scheme 2-2.^{143,144} Amine compound **32** was protected with di-tert-butyl dicarbonate to yield the the boc-protected amine **33**. Substitution of the hydroxyl group of **33** with a phthalamide group was carried out under Mitsunobu conditions to give the intermediate **34**. Next, the removal of the boc group yielded the amine **35**, which was coupled to chlorothienopyrimidine **7** to afford the intermediate **36**. In the final step, the deprotection of the phthalamide group of **36** afforded the targeted thienopyrimidine **31**. However, **31** did not show improved activity compared to **30**, suggesting the hydroxyl moiety is required for the anti-*H. pylori* activity.

Initial hits **1** and **2** also differ in their substitution patterns at -C5 and C6 of the thienopyrimidine ring. Compounds **37-40** were synthesized as described in Scheme 2-3. Commercially available chlorothienopyrimidines (**41-44**) were used in the coupling reaction with the optimized phenylglycinol sidechain to yield final compounds (**37-40**). The effect of substitution at these positions was then studied in combination with the optimized phenylglycinol 4-position sidechain. Compounds **37**, **38**, **39** and **40** (Table 2-4) were generated and their activities were compared to compound **28** (Table 2-3). Moving the phenyl ring to the 5-position (**39** and **40**) substantially reduced anti-*H. pylori* potency. The dimethyl analog **30** with a similar substitution pattern of compound **2**, showed a slight improvement over **2** but weaker activity than the 6-phenyl substituted analog **38**. **38** with a similar phenyl substitution pattern to series 3 compounds and an additional methyl substitution, demonstrates comparable activity to **28**, suggesting the phenyl ring at the 6-position optimal and 5-position can be hydrogen and methyl but not a larger phenyl group.

To determine the target of the thienopyrimidines, resistant *H. pylori* mutants were successfully isolated by serial passage on agar medium containing compound **25**. Upon whole genome sequencing, two spontaneous mutants were identified containing non-synonymous point mutations in *nuoD*. The resulting amino acid substitutions were T400I

Scheme 2-2. Synthesis of amine 31.

Reagents and conditions: (i) di-*tert*-butyl dicarbonate, Et_3N , DCM, RT, 2 h; (ii) phthalimide, DIAD, Ph_3P , DCM, RT, 4 h; (iii) 4 M HCl in dioxane, DCM, methanol, RT, 4 h; (iv) Et_3N , reflux, 6 h; (v) NH_2NH_2 , methanol, RT, 6 h.

Scheme 2-3. Synthesis of thienopyrimidine analogs with modifications at the 5 and 6-position.

Reagents and conditions: (i) Et_3N , 150°C , Microwave, 1 h, 89-93%.

Table 2-4. The structure-activity relationship of series 4 with substitutions at the 5 and 6-positions of the thienopyrimidine core.

<div style="text-align: center;"> </div>						
Compound	R ₁	R ₂	Log P	<i>H. pylori</i> IC ₅₀ ± SEM (µg/mL) SS1 strain ^a	Cytotoxicity IC ₅₀ ± SEM (µg/mL) FaDu Cells ^a	LLE ^b
37	Me—	Me—	4.05	0.25 ± 0.18	24.61	-1.84
38	Me—		5.45	0.01 ± 0.001	447.64	-4.51
39		Me—	5.24	10.24 ± 13.59	4.37	-1.7
40		H—	4.91	0.21 ± 0.16	60.69	-2.81

^a All IC₅₀ data are the mean of 3 measurements. ^b LLE = pIC₅₀ – log P.

and A402P (**Figure 2-2**). The gene, *nuoD* encodes for the D subunit of NADH:Quinone Oxidoreductase, also known as the respiratory Complex I (**Figure 2-3A**).^{109, 136-138} These results suggest that Complex I, a protein that catalyzes the first step of oxidative phosphorylation, is the putative target of thienopyrimidines.^{107, 139}

To further understand the inhibition of *H. pylori*'s Complex I with the thienopyrimidine **25**, a homology model of the wildtype subunits NuoD and the neighboring NuoB was generated using *Thermus thermophilus*' Complex I as a template.²⁰ Using Clustal Omega it was found that the NuoB subunit has 59% homology with *T. thermophilus*' complex I subunit Nqo6 and 42% homology with *T. thermophilus*' complex I subunit Nqo4. The T400I and A402P mutations in NuoD are found in the quinone binding pocket indicating the thienopyrimidine series bind in this pocket. In order to visualize the possible binding, the chiral thienopyrimidine **26** was docked into the known menaquinone binding pocket at the NuoB-NuoD interface (**Figure 2-3B**). This model suggests that thienopyrimidine **26** forms a hydrogen bond with the backbone of T400 and suggests the small molecule inhibits menaquinone binding. In support of the computational studies, the thienopyrimidine **26** IC₅₀ data shows toxicity to both wild type and the A402P mutant *H. pylori*. Further details of the target identification studies and the homology modeling are provided in the Experimental section of chapter II. To validate that the thienopyrimidines are on target, all analogs were tested against the mutant *H. pylori* strain (SS1 nuoD A402P). The IC₅₀ values against the mutant strain are provided in **Tables A-1** and **A-2**. The thienopyrimidines are found generally inactive against the mutant strain, further supporting that Complex I is the target of the series.

Given compound **25** demonstrates high *in vitro* potency (H.p. IC₅₀ = 0.006 µg/ml, **Table 2-3**), we evaluated the *in vitro* ADME and *in vivo* pharmacokinetic properties.²²⁻²⁴ The results of these studies are provided in **Tables A-3** and **A-4**, respectively. Compound **25** showed good microsomal stability ($t_{1/2}$ = 3.8 hr) and permeability (Ave Pe = 239.5 x 10⁻⁶ cm/s) but it is highly bound to human plasma protein (HPPB % = 99). **25** showed good *in vivo* exposure (AUC = 31907.71 hr*ng/mL, **Table A-3**) at 50 mg/kg, i.p. dose. The drug concentration of **25** in the plasma was above its IC₅₀ value for more than 8 hours. With good plasma exposure and cytotoxicity profile, we proceeded to test **25** in the *H. pylori* murine infection model.^{133,134} Further details are provided in (**Figure A-1**). Compared to the BMT (Bismuth, Metronidazole and Tetracycline) triple therapy as control, **25** did not show efficacy at 50 mg/kg (OG), 50 mg/kg (i.p) or 25 mg/kg (i.p).¹³⁵ To investigate the lack of *in vivo* efficacy, we tested compound **25** in the *ex vivo* model of *H. pylori* infection. Ten mice were infected with *H. pylori* SS1 strain and sacrificed after three days to harvest the stomachs.^{134,140} In a 12 well plate, one half of each stomach was placed in Brucella-FBS medium containing compound **25** and the other half was placed in Brucella-FBS medium only and treated as control. Further details of the *ex vivo* experiment are described in the experimental section of chapter II. As described in

	391	409
mut2_genome	AVTVIGSTNPVFGEVDR	
mut2_PCR	AVTVIGSTNPVFGEVDR	
mut1_genome	AVTVIGSINAVFGEVDR	
mut1_PCR	AVTVIGSINAVFGEVDR	
WT_nuoD_genome	AVTVIGSTNAVFGEVDR	
	***** * *****	

Figure 2-2. Sequence alignment of the wildtype *nuoD* and two *H. pylori* mutant *nuoD*.

Resistant mutants were generated by exposing *H. pylori* to compound **25** on agar plate. Whole genome sequencing was performed, and single nucleotide polymorphism analysis identified two spontaneous mutants, which had non-synonymous point mutations in *nuoD*. The resulting amino acid changes were Threonine to Isoleucine (T400I) and Alanine to Proline (A402P). *nuoD* encodes for the subunit D of NADH:Quinone Oxidoreductase also known as Complex I, a membrane protein that catalyzes the first step of oxidative phosphorylation. Complex I is the putative target of the thienopyrimidine.

A.

B.

Figure 2-3. The homology model of *H. pylori*'s respiratory Complex I, the putative target of the thienopyrimidines.

(A) The structure of Complex I with NuoD colored in dark red. The homology model was generated using the crystal structure of *Thermus Thermophilus*' Complex I. The thienopyrimidines are predicted to bind at the interface of NuoD and NuoB. (B) The lead compound **26** docked in the proposed binding pocket of Complex I. The quinone binding residues are labeled in purple and the substituted amino acids in resistant mutants (T400I and A402P) against the thienopyrimidine **26** are labeled in blue. According to the homology model, the changed amino acids are located near the quinone binding pocket, suggesting **26** inhibits the endogenous quinone from binding to Complex I.

Figure 2-4, **25** substantially eradicates *H. pylori*, with 6 stomachs found below the limit of detection (**Figure 2-4**). Compound **25** eradicates *H. pylori* in the *ex vivo* settings but does not show *in vivo* efficacy. This discrepancy suggests that not enough of **25** is getting to the site of infection and the optimization of the pharmacokinetic properties is required for the series to demonstrate *in vivo* efficacy.

Conclusion

In conclusion, we have synthesized 27 analogs and established the anti-*H. pylori* SAR at the 4, 5 and 6 position of the thienopyrimidine core. These efforts have generated potent compounds, including the lead compound **25**, with the IC₅₀ of 0.006 µg/mL. Although **25** does not show *in vivo* efficacy, it eradicates *H. pylori* in the *ex vivo* model. The mode of action studies identified *H. pylori*'s respiratory Complex I as the putative target of the thienopyrimidines, with the amino acid changes found in the NuoD subunit. Complex I is located in the plasma membrane of bacteria and it catalyzes the first step of oxidative phosphorylation. Targeting oxidative phosphorylation has been done safely in the clinic, as exemplified by metformin, a drug used for the treatment of diabetes.¹⁴¹ Additionally, targeting oxidative phosphorylation is becoming an attractive approach in the treatment of relapsed/refractory AML and solid tumors.¹⁴² Recent knockout mutagenesis studies suggest that *nuoD* is an essential gene for *H. pylori* but not for the majority of other bacteria, indicating that *H. pylori* can be targeted selectively without perturbation the host microbiota.^{16,31} These examples suggest that Complex I is a worthwhile target in eradicating *H. pylori*. **25** is a promising lead compound that is worth further optimization to realize *in vivo* efficacy in the *H. pylori* infected mouse model.

Figure 2-4. Compound 25 eradicates *H. pylori* in the *ex vivo* efficacy experiment. Ten mice were infected with *H. pylori*. After 3 days, stomachs were harvested and cut in half. In a 12 well plate, one half was placed in Brucella-FBS medium and the other half in Brucella-FBS medium with 0.6 $\mu\text{g/mL}$ of compound **25**. After incubation and homogenization, diluted samples were plated for colony counting. **25** eradicates *H. pylori* to below the limit of detection in 6 stomachs.

CHAPTER 3. ADME OPTIMIZATION OF THE THIENOPYRIMIDINES AGAINST HELICOBACTER PYLORI

Introduction

The rate of successful treatment of *H. pylori* infections, with the clarithromycin triple therapy, is around 75%.¹ The triple therapy, which consists of a proton pump inhibitor and two broad-spectrum antibiotics such as clarithromycin and amoxicillin, is increasingly becoming ineffective due to the rise of resistance to these antibiotics.^{26,27,31} The World Health Organization lists *H. pylori* as a high priority drug resistant bacterium.⁷ In a recent study, 22% of patients tested were not cured by either the triple therapy or the quadruple therapy with Bismuth added.²⁷ The triple therapy is also associated with poor patient compliance due to side effects such as diarrhea, derived from the effect of these broad-spectrum antibiotics on the host microbiota.¹¹ Therefore, a novel, narrow-spectrum drug is needed for the treatment of *H. pylori* infections, to address the challenges of drug resistance against anti-*H. pylori* drugs and their side effects.

Advances in whole genome sequencing and highthrough-put screening approaches have provided opportunities to discover novel chemical matter against *H. pylori*. We have previously reported a series of thienopyrimidines that were developed from two hits, discovered through a high through-put screen of 219,197 compounds. Subsequent structure-activity relationship studies of the thienopyrimidines against *H. pylori*, compound **25** (**Figure 3-1A**) emerged with a high potency (*H. pylori* IC₅₀ = 0.006 µg/mL) and tolerable cytotoxicity against the human FaDu cells (Cytotox IC₅₀ = 131 µg/mL). Mode of action studies with the lead compound **25** were performed by the generation and sequencing of resistant mutants. These studies revealed the respiratory Complex I as the putative target of the series, with amino acid changes found in the NuoD subunit. NuoD has been previously identified as the target of a series of the benzimidazole compounds by researchers at AstraZeneca and it was found to be essential for *H. pylori* but not for most of bacteria including *E. coli*.^{105,106} Therefore, NuoD is an attractive target, in eradicating *H. pylori* while preserving the gut microbiota. Complex I, also known as NADH:Quinone Oxidoreductase, catalyzes the first step of oxidative phosphorylation.¹³⁹

The lead compound **25** eradicates *H. pylori* in the *ex vivo* model of *H. pylori* infection. However, **25** lacked *in vivo* efficacy in the *H. pylori* murine model at 50 mg/kg by oral gavage; suggesting that further optimization of pharmacokinetic properties of the series is required. An examination of the *in vivo* pharmacokinetic properties, **25** shows good exposure (AUC: 31,907 hr*ng/mL at 50 mg/kg OG, **Figure 3-1A**) and a long high half-life in liver microsomes (t_{1/2} = 3.8 hr, **Figure 3-1A**) suggesting that hepatic metabolism was not the limiting factor. However, **25** demonstrates a relatively high volume of distribution (V_{ss} = 6.28 L, **Figure 3-1A**), suggesting **25** is being trapped in fatty tissues despite good exposure.¹⁴⁵ **25** also shows poor solubility (Sol: 0.1 µM,

Figure 3-1. Lead compound 25 and targeted moieties to improve the ADME properties.

(A) ^a*in vivo* pharmacokinetic properties of **25** at 50 mg/Kg i.p. and ^bADME properties of **25**. (B) Chemical modifications on targeted moieties of **25**, to improve the ADME properties of the series.

Figure 3-1A) and is highly bound to plasma protein (HPPB% = 99.3, **Figure 3-1A**), reducing the amount of free drug able to reach the site of infection. These limitations are compounded by the hard to reach biological niche of *H. pylori*, found in the glands of the stomach and in the mucus layer.

To tackle these shortcomings, in this chapter we look to introduce chemical modifications that will reduce the lipophilicity and increase the solubility while maintaining selective anti-*H. pylori* activity and resistance to hepatic metabolism of **25**. In this chapter we explore the optimization of **25** through targeted synthesis and examination of the anti-*H. pylori* activity and ADME properties of four subseries (**Figure 3-1B**): (i) We replace metabolically labile and solubility limiting phenyl ring substituted at the 6-position of the thienopyrimidine scaffold with pyridinyl, pyrazolyl and methyl motifs; (ii) Through a scaffold hopping exercise, the introduction of core modifications to improve the solubility and reduce the tendency of drug to bind to plasma protein are examined;¹⁴⁹ (iii) The introduction of primary amine motif to the side chain of **25**, increase Gram-negative drug entry and increase the distribution in the body fluids is also tested;^{146,147} (iv) to reduce drug clearance and compensate for changes elsewhere in the ring, introduce blocking modifications such as para-fluoro-substitution of the 6-phenyl ring of the thienopyrimidine core are also examined.¹⁴⁸ Through analysis of the microbiological and pharmacological properties of the new analogs, new lead candidates have emerged suitable for further evaluations.

Materials and Methods

Synthesis of Amine 46

tert-butyl (2-hydroxy-1-(4-methoxyphenyl)ethyl)carbamate (60). Di-*tert*-butyl dicarbonate (0.447 g, 2.047 mmol) was added to a solution of 2-hydroxy-1-(4-methoxyphenyl)ethan-1-aminium chloride **59** (0.347 g, 1.706 mmol) and triethylamine (0.286 mL, 2.047 mmol) in DCM (5.6 mL). The reaction mixture was stirred for 2 h at room temperature. The mixture was concentrated and purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-80% ethyl acetate in hexanes). Pure fractions were evaporated to afford **60** as a white solid.

tert-butyl (2-(1,3-dioxoisindolin-2-yl)-1-(4-methoxyphenyl)ethyl)carbamate (61). DIAD (0.141 mL, 0.729 mmol) was added dropwise at room temperature, to a solution of **60** (0.150 g, 0.561 mmol), phthalimide (0.107 g, 0.729 mmol) and Ph₃P (0.191 g, 0.729 mmol) in THF (.6 mL). The resulting mixture was stirred for 16 h at room temperature. The mixture was extracted with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-60% ethyl acetate in hexanes), to afford **61** as a yellow solid.

2-(2-amino-2-(4-methoxyphenyl)ethyl)isoindoline-1,3-dione (62) and 2-(2-(4-methoxyphenyl)-2-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)ethyl)isoindoline-1,3-dione (63). HCl (4 M in 1,4-dioxane, 0.9 mL, 3.6 mmol) was added to a solution of **61** (0.300 g, 0.757 mmol) in a mixture of DCM (2 mL) and methanol (1 mL), and the resulting mixture was stirred for 16 h at room temperature. The mixture was subjected to acid-base extraction with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **62** (yellow oil) was used in the next step without further purification. **63** was synthesized following the general procedure for amination of 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

1-(4-methoxyphenyl)-N1-(6-phenylthieno[2,3-*d*]pyrimidin-4-yl)ethane-1,2-diamine (46). Compound **63** (0.130 g, 0.257 mmol) was dissolved in methanol and a 80 % hydrazine hydrate solution (0.04 mL, 0.642 mmol) was added dropwise to the solution at 0°C. The reaction mixture was stirred for 16 h at room temperature. The mixture was extracted with Ethyl Acetate/Water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 2-20 % methanol in ethyl acetate) to afford **46** as a white solid.

1-(4-methoxyphenyl)-N1-(6-phenylthieno[2,3-*d*]pyrimidin-4-yl)ethane-1,2-diamine (46). ¹H NMR (DMSO, 400 MHz) δ 8.32 (d, *J* = 0.7 Hz, 1H), 8.03 (s, 1H), 7.77 – 7.67 (m, 2H), 7.51 (dd, *J* = 8.3, 7.0 Hz, 2H), 7.45 – 7.35 (m, 3H), 6.97 – 6.88 (m, 2H), 5.65 (dd, *J* = 10.6, 4.4 Hz, 1H), 3.70 (s, 3H), 3.32 (dd, *J* = 13.1, 10.6 Hz, 1H), 3.23 (dd, *J* = 13.1, 4.5 Hz, 1H). MS (ESI): *m/z* 377 (M⁺+H).

Synthesis of Thienopyrimidine Analog 45

45 was synthesized following the general procedure for amination of 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

(R)-3-(4-methoxyphenyl)-3-((6-phenylthieno[2,3-*d*]pyrimidin-4-yl)amino)propan-1-ol (45). ¹H NMR (DMSO, 400 MHz): δ 8.27 (d, *J* = 4.1 Hz, 1H), 8.19 – 8.11 (m, 2H), 7.74 – 7.64 (m, 2H), 7.52 (q, *J* = 6.4, 6.0 Hz, 2H), 7.41 (d, *J* = 8.8 Hz, 1H), 7.36 – 7.30 (m, 2H), 6.91 – 6.84 (m, 2H), 5.46 (d, *J* = 7.7 Hz, 1H), 4.56 (t, *J* = 4.7 Hz, 1H), 3.71 (d, *J* = 3.2 Hz, 2H), 3.46 (dd, *J* = 13.0, 6.6 Hz, 2H), 2.09 (d, *J* = 5.1 Hz, 1H), 2.01 – 1.86 (m, 1H); MS (ESI): *m/z* 392.3 (M⁺+H).

Synthesis of Primary Amine 47

Benzyl (R)-(3-hydroxy-1-(4-methoxyphenyl)propyl)carbamate (65). Benzyl chloroformate chloride (226 μL, 1.584 mmol) was added to a solution of **64** (261 mg, 1.440 mmol) and trimethylamine (0.504 mL, 3.60 mmol) in THF (7 mL). The reaction mixture was stirred for 14 h at room temperature. The reaction was concentrated and extracted with ethyl acetate and water. The organic phase was washed with brine, dried

(Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-60% ethyl acetate in hexanes), to afford **65** as a white solid.

Benzyl (R)-(3-(1,3-dioxoisindolin-2-yl)-1-(4-methoxyphenyl)propyl)carbamate (66). DIAD (152 mL, 0.785 mmol) was added dropwise at room temperature, to a solution of **65** (0.225 g, 0.7135 mmol), phthalimide (0.115 g, 0.785 mmol) and Ph₃P (0.206 g, 0.785 mmol) in THF (3.5 mL). The resulting mixture was stirred for 16 h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo. The residue was purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-60 ethyl acetate in hexanes), to afford **66** as a yellow solid.

Benzyl (R)-(3-amino-1-(4-methoxyphenyl)propyl)carbamate (67). Compound **66** (0.317 g, 0.713 mmol) was dissolved in methanol and a 80 % hydrazine hydrate solution (0.109 mL, 1.783 mmol) was added dropwise to the solution at 0°C. The reaction mixture was stirred for 16 h at room temperature. The mixture was subjected to acid-base extraction with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **67** (colorless oil) was used in the next reaction without further purification.

Benzyl (R)-(3-(((11-methyl)(11-oxidaneryl)boraneryl)amino)-1-(4-methoxyphenyl)propyl)carbamate (68). Di-tert-butyl dicarbonate (0.143 g, 0.657 mmol) was added to a solution of **67** (0.172 g, 0.547 mmol) and trimethylamine (0.153 mL, 1.094 mmol) in DCM (3 mL). The reaction mixture was stirred for 2 h at room temperature. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue **68** was used in the next step without further purification.

(R)-N1-(((11-methyl)(11-oxidaneryl)boraneryl)-3-(4-methoxyphenyl)propane-1,3-diamine (69). Pd/C (0.200 g, 10% wet) was added to a solution of **68** (0.137 g, 0.331 mmol) in methanol (8 mL). The reaction was stirred under hydrogen for 3h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. The residue **69** was used in the next step without further purification.

(R)-N3-(((11-methyl)(11-oxidaneryl)boraneryl)-1-(4-methoxyphenyl)-N1-(6-phenylthieno[2,3-d]pyrimidin-4-yl)propane-1,3-diamine (70). 4-chloro-6-phenylthienopyrimidine **7** (0.45 g, 0.168 mmol), amine **69** (0.61 g, 0.218 mmol), and triethylamine (0.36 mL, 0.254 mmol) were dissolved in ethanol and the mixture was heated at 115 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 12-60% ethyl acetate in hexanes) to afford **70** as a yellow solid.

(R)-1-(4-methoxyphenyl)-N1-(6-phenylthieno[2,3-d]pyrimidin-4-yl)propane-1,3-diamine (47). HCl (4M in 1,4-dioxane, 0.073 mL, 0.292 mmol) was added to a solution

of **70** (0.053 g, 0.215 mmol) in a mixture of DCM (1 mL) and methanol (0.4 mL), and the resulting mixture was stirred for 16h at room temperature. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and evaporated in vacuo to afford **47** as a yellow solid.

(R)-1-(4-methoxyphenyl)-N1-(6-phenylthieno[2,3-d]pyrimidin-4-yl)propane-1,3-diamine (47). ¹H NMR (DMSO, 500 MHz): δ 8.41 (s, 1H), 8.32 (s, 1H), 8.11 (s, 1H), 7.73 (d, 2H, J = 8.1 Hz), 7.53 (t, 2H, J = 7.45 Hz), 7.45-7.39 (m, 2H), 6.93 (d, 2H, J = 8.75 Hz), 5.45-5.43 (m, 1H), 4.79-4.76 (m, 2H), 3.73 (s, 3H), 2.97-2.89 (m, 1H), 2.79-2.75 (m, 1H), 1.21-1.18 (m, 2H); MS (ESI): *m/z* 391 (M⁺+H).

Synthesis of Amine 48

Tert-butyl (R)-(3-hydroxy-1-(4-methoxyphenyl)propyl)carbamate (71). Di-*tert*-butyl dicarbonate (1.98 g, 9.10 mmol) was added to a solution of **64** (1.5 g, 8.28 mmol) and trimethylamine (1.27 mL, 9.10 mmol) in DCM (40 mL). The reaction mixture was stirred for 2 h at room temperature. The mixture was concentrated and purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-80% ethyl acetate in hexanes) to afford **71** as a white solid.

(R)-3-((tert-butoxycarbonyl)amino)-3-(4-methoxyphenyl)propyl methanesulfonate (72). Methanesulfonyl chloride (0.825 mL, 10.66 mmol) was added to a solution of **71** (1 g, 3.55 mmol) and trimethylamine (1.487 mL, 10.66 mmol) at 0°C. The reaction mixture was stirred for 3h at room temperature. The mixture was concentrated and purified by flash column chromatography (Isolera 1, Biotage, 10 g size, elution gradient 12-80% ethyl acetate in hexanes) to afford **72** as a yellow solid.

Tert-butyl (R)-(3-(dimethylamino)-1-(4-methoxyphenyl)propyl)carbamate (73). In a microwave tube, compound **72** (0.075 g, 0.21 mmol) and tetrabutylammonium (0.01 g, 0.042 mmol) were dissolved with dimethylamine (2M in THF, 2 mL) and subjected to microwave heating at 150°C for 0.5 h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **73** (bright red oil) was used in the next step without further purification.

(R)-1-(4-methoxyphenyl)-N3,N3-dimethylpropane-1,3-diamine (74). HCl (4 M in 1,4-dioxane, 0.422 mL, 1.688 mmol) was added to a solution of **73** (0.13 g, 0.422 mmol) in a mixture of DCM (2 mL) and methanol (0.8 mL), and the resulting mixture was stirred for 16h at room temperature. The mixture was subjected to acid-base extraction with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **74** (colorless oil) was used in the next step without further purification.

(R)-1-(4-methoxyphenyl)-N3,N3-dimethyl-N1-(6-phenylthieno[2,3-d]pyrimidin-4-yl)propane-1,3-diamine (48). 4-Chloro-6-phenyl-thienopyrimidine **7** (0.048 g, 0.195

mmol), amine **74** (0.05 g, 0.204 mmol), and triethylamine (0.056 mL, 0.408 mmol) were dissolved in ethanol and the mixture was heated at 115 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **48** as yellow solid.

(R)-1-(4-methoxyphenyl)-N3,N3-dimethyl-N1-(6-phenylthieno[2,3-d]pyrimidin-4-yl)propane-1,3-diamine (48). ¹H NMR (MeOD, 500 MHz) δ 8.27 (d, *J* = 1.1 Hz, 1H), 7.90 (s, 1H), 7.70 (dt, *J* = 8.4, 1.7 Hz, 2H), 7.45 (dd, *J* = 8.5, 7.1 Hz, 2H), 7.41 – 7.34 (m, 3H), 6.92 – 6.88 (m, 2H), 5.44 (t, *J* = 7.3 Hz, 1H), 3.80 – 3.75 (m, 3H), 2.48 (ddd, *J* = 12.6, 9.9, 5.3 Hz, 1H), 2.37 (ddd, *J* = 12.6, 9.9, 6.0 Hz, 1H), 2.28 (s, 6H), 2.21 – 2.09 (m, 2H); MS (ESI): *m/z* 419 (M⁺+H).

Synthesis of Thienopyrimidine Analog 49

49 was synthesized following the general procedure for the amination 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

(R)-3-((6-phenylthieno[2,3-d]pyrimidin-4-yl)amino)-3-(pyridin-4-yl)propan-1-ol (49). ¹H NMR (MeOD, 500 MHz) δ 8.54 – 8.44 (m, 2H), 8.26 (d, *J* = 2.7 Hz, 1H), 7.96 (s, 1H), 7.81 – 7.70 (m, 2H), 7.59 – 7.51 (m, 2H), 7.49 (dd, *J* = 8.5, 7.0 Hz, 2H), 7.44 – 7.37 (m, 1H), 5.65 (dd, *J* = 9.2, 5.5 Hz, 1H), 3.81 – 3.66 (m, 2H), 2.21 (qdt, *J* = 13.9, 7.8, 5.5 Hz, 2H); MS (ESI): *m/z* 363 (M⁺+H).

Synthesis of Thienopyrimidine and Pyrrolopyrimidine Analogs with Modifications at the 6-position

2-((6-bromothieno[2,3-d]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (76a). 4-Chloro-6-bromo-thienopyrimidine **75a** (0.025 mg, 0.100 mmol), amine **59** (0.050 mg, 0.299 mmol) and trimethylamine (0.042 mL, 0.299 mmol) were dissolved in ethanol and the mixture was heated at 115 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **76a** (red solid, with the pyrrolopyrimidine) was used in the next reaction without further purification.

2-(4-methoxyphenyl)-2-((6-(pyridin-4-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (50). Compound **76a** (0.08 g, 0.210 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and compound **77a** (0.039 g, 0.316 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO₃ (0.083 g, 0.993 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified

by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **50** as yellow solid.

2-(4-methoxyphenyl)-2-((6-(pyridin-4-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (50). ¹H NMR (MeOD, 400 MHz) δ 8.31 – 8.18 (m, 1H), 8.03 – 7.93 (m, 1H), 7.81 – 7.68 (m, 2H), 7.52 – 7.45 (m, 2H), 7.43 – 7.36 (m, 3H), 6.92 (ddd, J = 8.5, 3.2, 1.5 Hz, 2H), 5.52 (s, 1H), 3.94 (dd, J = 5.8, 3.5 Hz, 2H), 3.82 – 3.70 (m, 3H); MS (ESI): m/z 379.4 ($M^+ + H$).

2-(4-methoxyphenyl)-2-((6-(pyridin-3-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (51). Compound **76a** (0.1 g, 0.263 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and compound **77b** (0.048 g, 0.394 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO₃ (0.104 g, 1.238 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **51** as yellow solid.

2-(4-methoxyphenyl)-2-((6-(pyridin-3-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (51). ¹H NMR (DMSO, 500 MHz) δ 8.95 (d, J = 2.5 Hz, 1H), 8.60 (dd, J = 4.8, 1.5 Hz, 1H), 8.31 (s, 1H), 8.35 (s, 1H), 8.23 (d, J = 8.0 Hz, 1H), 8.08 (dt, J = 8.0, 2.0 Hz, 1H), 7.40 – 7.32 (m, 2H), 6.93 – 6.86 (m, 2H), 5.41 (td, J = 7.9, 5.3 Hz, 1H), 5.04 (s, 2H), 3.72 (s, 4H); MS (ESI): m/z 379.2 ($M^+ + H$).

2-(4-methoxyphenyl)-2-((6-(pyridin-2-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (52). Compound **76a** (0.1 g, 0.263 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and compound **77c** (0.048 g, 0.394 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO₃ (0.104 g, 1.238 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **52** as yellow solid.

2-(4-methoxyphenyl)-2-((6-(pyridin-2-yl)thieno[2,3-d]pyrimidin-4-yl)amino)ethan-1-ol (52). ¹H NMR (DMSO, 400 MHz) δ 8.95 (d, J = 2.4 Hz, 1H), 8.60 (dd, J = 4.8, 1.5 Hz, 1H), 8.33 (d, J = 12.6 Hz, 2H), 8.20 (d, J = 8.0 Hz, 1H), 8.08 (ddd, J = 8.0, 2.5, 1.5 Hz, 1H), 7.36 (d, J = 8.6 Hz, 2H), 6.92 – 6.87 (m, 2H), 5.41 (q, J = 7.5 Hz, 1H), 5.02 (t, J = 5.7 Hz, 1H), 3.72 (s, 5H); MS (ESI): m/z 379.4 ($M^+ + H$).

2-((6-(1H-pyrazol-4-yl)thieno[2,3-d]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (53). Compound **76a** (0.1 g, 0.263 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and compound **77d** (0.044 g, 0.394 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO₃ (0.104 g, 1.238 mmol) was added and the mixture was

heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **53** as yellow solid.

2-((6-(1H-pyrazol-4-yl)thieno[2,3-d]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (53). ¹H NMR (DMSO, 400 MHz) δ 8.22 (d, *J* = 1.6 Hz, 1H), 8.08 (d, *J* = 7.9 Hz, 1H), 7.84 (s, 1H), 7.39 – 7.30 (m, 2H), 6.92 – 6.83 (m, 2H), 5.36 (q, *J* = 7.8 Hz, 1H), 5.07 (s, 1H), 3.72 (d, *J* = 1.4 Hz, 5H); MS (ESI): *m/z* 368.3 (M⁺+H).

Synthesis of Thienopyrimidine Analog 54

54 was synthesized following the general procedure for the amination 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

2-((5,6-dimethylthieno[2,3-d]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (54). ¹H NMR (CDCl₃, 400 MHz) δ 8.33 (s, 1H), 7.35 – 7.28 (m, 2H), 6.92 (dd, *J* = 8.4, 1.3 Hz, 2H), 6.15 (d, *J* = 5.8 Hz, 1H), 5.35 (q, *J* = 5.5 Hz, 1H), 4.06 – 3.96 (m, 2H), 3.81 (d, *J* = 1.0 Hz, 3H), 2.49 – 2.38 (m, 6H). MS (ESI): *m/z* 330.01 (M⁺+H).

Synthesis of Fluorophenyl Thienopyrimidine Analog 55

Ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate (79). In a microwave tube, ethyl 3-cyanoacetate **4** (0.693 mL, 6.52 mmol) was added to a solution of 2-(4-fluorophenyl)acetaldehyde **78** (0.804 g, 6.52 mmol), sulfur (0.23 g, 7.17 mmol) and morpholine (0.651 mL, 7.54 mmol) in ethanol (20 mL). The resulting mixture was submitted to microwave heating for 30 min at 70°C. After cooling, the mixture was filtered, and the filtrate was poured in water. The precipitate was collected, dried and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient 12-60% ethyl acetate in hexanes) to afford **79** as a yellow solid.

Ethyl 2-amino-5-(4-fluorophenyl)thiophene-3-carboxylate (79). ¹H NMR (MeOD, 500 MHz): δ 7.45-7.41 (m, 2H), 7.14 (s, 1H), 7.08-7.03 (m, 2H), 4.58 (s, 2H), 4.30-4.24 (q, 2H, *J* = 9.05 Hz), 1.37-1.29 (t, 3H, *J* = 8.95 Hz); MS (ESI): *m/z* 266 (M⁺+H).

6-(4-fluorophenyl)thieno[2,3-d]pyrimidin-4(3H)-one (80). Compound **79** (1.15 g, 4.33 mmol) was dissolved in excess formamide and the resulting mixture was heated for 16 h at 160°C. The mixture was concentrated in vacuo and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient 12-60% ethyl acetate in hexanes) to afford **80** as a red solid.

6-(4-fluorophenyl)thieno[2,3-d]pyrimidin-4(3H)-one (80). ¹H NMR (DMSO, 500 MHz): δ 8.41 (s, 1H), 7.85-7.81 (m, 2H), 7.78 (s, 1H), 7.33-7.27 (m, 2H); MS (ESI): *m/z* 247 (M⁺+H).

4-chloro-6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidine (81). Compound **80** (0.32 g, 0.13 mmol) and N,N-dimethylaniline (0.001 mL, 0.009 mmol) were dissolved in phosphoryl trichloride (0.5 mL, 8.12 mmol) and the mixture was heated for 16 h to 95 °C. The mixture was concentrated in vacuo and purified by flash column chromatography (Isolera 1, Biotage, 25 g size, elution gradient of 12-60% ethyl acetate in hexanes) to afford **81** as a yellow solid.

4-chloro-6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidine (81). ¹H NMR (CDCl₃, 500 MHz): δ 8.82 (s, 1H), 7.74-7.69 (m, 2H), 7.52 (s, 1H), 7.22-7.16 (m, 2H); MS (ESI): *m/z* 265 (M⁺+H).

In the last step, **55** was synthesized following the general procedure for the amination 4-chloro-6-phenylthieno[2,3-*d*]pyrimidine.

2-((6-(4-fluorophenyl)thieno[2,3-*d*]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (55). ¹H NMR (CDCl₃, 400 MHz) δ 8.45 (s, 1H), 7.66 – 7.59 (m, 2H), 7.35 (d, *J* = 8.7 Hz, 2H), 7.13 (t, *J* = 8.6 Hz, 3H), 6.94 (d, *J* = 8.7 Hz, 2H), 5.72 (d, *J* = 6.2 Hz, 1H), 5.40 (d, *J* = 4.0 Hz, 1H), 4.13 – 4.03 (m, 3H), 3.82 (s, 3H); MS (ESI): *m/z* 396.2 (M⁺+H).

Synthesis of Pyrrolopyrimidine and Quinazoline Analogs with Modification at the 6-position

2-((6-bromo-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (76b). 6-Bromo-4-chloro-7H-pyrrolo[2,3-*d*]pyrimidine **75b** (0.070 mg, 0.301 mmol), amine **59** (0.076 mg, 0.452 mmol) and trimethylamine (0.126 mL, 0.904 mmol) were dissolved in ethanol and the mixture was heated at 115 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The resulting residue **76b** was used in the next reaction without further purification.

2-(4-methoxyphenyl)-2-((6-phenyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)ethan-1-ol (56). Compound **76b** (0.081 g, 0.263 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and compound **77e** (0.039 g, 0.316 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO₃ (0.083 g, 0.993 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-20% methanol in ethyl acetate) to afford **56** as yellow solid.

2-(4-methoxyphenyl)-2-((6-phenyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)ethan-1-ol (56). ¹H NMR (DMSO, 500 MHz) δ 12.11 (s, 1H), 8.08 (s, 1H), 7.80 (d, *J* = 7.8 Hz, 2H), 7.45 (t, *J* = 7.6 Hz, 2H), 7.35 (d, *J* = 8.3 Hz, 2H), 7.30 (t, *J* = 7.4

Hz, 1H), 7.15 (s, 1H), 6.91 – 6.83 (m, 2H), 5.36 (q, $J = 7.4$ Hz, 1H), 4.95 (s, 1H), 3.81 – 3.64 (m, 5H); MS (ESI): m/z 361.4 ($M^+ + H$).

2-((7-bromoquinazolin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (83). 7-bromo-4-chloroquinazoline **82** (0.2 g, 0.821 mmol), amine **59** (0.208 g, 1.232 mmol) and trimethylamine (0.573 mL, 4.11 mmol) were dissolved in ethanol and the mixture was subjected to microwave heating at 150 °C for 1h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The resulting residue **83** was used in the next reaction without further purification.

2-(4-methoxyphenyl)-2-((7-phenylquinazolin-4-yl)amino)ethan-1-ol (57). Compound **83** (0.1 g, 0.267 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and phenylboronic acid **77e** (0.049 g, 0.401 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO_3 (0.185 g, 1.336 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-100% ethyl acetate in hexanes) to afford **57** as white solid.

2-(4-methoxyphenyl)-2-((7-phenylquinazolin-4-yl)amino)ethan-1-ol (57). ^1H NMR (DMSO, 500 MHz) δ 8.57 (d, $J = 8.6$ Hz, 1H), 8.44 – 8.40 (m, 2H), 8.21 (s, 1H), 7.93 – 7.87 (m, 2H), 7.87 – 7.83 (m, 2H), 7.53 (dd, $J = 8.3, 7.0$ Hz, 2H), 7.48 – 7.43 (m, 1H), 7.40 – 7.34 (m, 2H), 6.90 – 6.85 (m, 2H), 5.48 (td, $J = 8.2, 5.2$ Hz, 1H), 3.84 (dd, $J = 11.2, 8.6$ Hz, 1H), 3.71 (s, 4H). MS (ESI): m/z 372.4 ($M^+ + H$).

Synthesis of Quinazoline Analog 58

2-((6-bromoquinazolin-4-yl)amino)-2-(4-methoxyphenyl)ethan-1-ol (81). 6-bromo-4-chloroquinazoline **75c** (0.200 g, 0.821 mmol), amine **59** (0.208 g, 1.232 mmol) and trimethylamine (0.573 mL, 4.11 mmol) were dissolved in ethanol and the mixture was subjected to microwave heating at 150 °C for 1h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The resulting residue **76c** was used in the next reaction without further purification.

2-(4-methoxyphenyl)-2-((6-phenylquinazolin-4-yl)amino)ethan-1-ol (58). Compound **76c** (0.1 g, 0.267 mmol), tetraphenyl palladium (0.005 g, 0.0012 mmol) and phenylboronic acid **77e** (0.049 g, 0.401 mmol) were dissolved in dioxane (4 mL). The solution was bubbled with nitrogen for 15 min. Then, an aqueous solution of NaHCO_3 (0.185 g, 1.336 mmol) was added and the mixture was heated at 75 °C for 14h. The mixture was extracted with ethyl acetate/water and the organic phase was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column

chromatography (Isolera 1, Biotage, 10 g size, elution gradient of 0-100% ethyl acetate in hexanes) to afford **58** as yellow solid.

2-(4-methoxyphenyl)-2-((6-phenylquinazolin-4-yl)amino)ethan-1-ol (58). ^1H NMR (500 MHz, DMSO) δ 8.77 (d, $J = 2.1$ Hz, 1H), 8.53 (d, $J = 7.9$ Hz, 1H), 8.40 (s, 1H), 8.15 (s, 1H), 8.11 (dd, $J = 8.6, 2.0$ Hz, 1H), 7.92 – 7.82 (m, 2H), 7.78 – 7.71 (m, 1H), 7.56 (t, $J = 7.8$ Hz, 2H), 7.44 (dd, $J = 8.2, 6.5$ Hz, 1H), 7.41 – 7.32 (m, 2H), 6.93 – 6.82 (m, 2H), 5.51 (td, $J = 8.2, 5.2$ Hz, 1H), 5.02 (s, 1H), 3.85 (dd, $J = 11.2, 8.6$ Hz, 1H), 3.81 – 3.61 (m, 4H); MS (ESI): m/z 372.3 ($\text{M}^+ + \text{H}$).

Results and Discussion

Chemistry

The synthesis of the amine compounds **46** and **31** (was carried out as described in **Scheme 3-1**. Starting from the amino-2-(4-methoxyphenyl)ethan-1-ol **59**, Boc-protection of the free amine gave alcohol **60**. Mitsunobu conditions were then used to introduce the targeted terminal amine as a phthalimide group to afford the intermediate **61**. After Boc-deprotection with HCl, **62** was reacted with 4-chloro, 6 phenyl thienopyrimidine **62** to give the substituted thienopyrimidine **63**, deprotection of the phthalimide group in the presence of hydrazine afforded **46**. The second targeted amine, compound **47**, was synthesized using a similar strategy as described in **Scheme 3-2**. Starting from (R)-3-amino-3-(4-methoxyphenyl)propanol **64**, amine protection with the benzyl chloroformate afforded **65**. Then, a phthalimide group was again introduced under mitsunobu conditions, to yield intermediate **66**. Cbz-protection of the (R)-3-amino-3-(4-methoxyphenyl)propanol **64**, from the previous step, prevents the attack of amine to the ketone of the phthalimide group that results in the cyclized side product. Deprotection of the phthalimide and subsequent protection of the free amine **67** with di-tert-butyl dicarbonate gave compound **68**. Deprotection reaction of benzyl chloroformate (Cbz) yielded amine **69**, which was coupled to 4-chloro, 6-phenylthienopyrimidine **7** to afford **70**. The targeted amine compound **47** was achieved following the final Boc-deprotection reaction of **70** in the presence of HCl.

To examine the SAR of **47**, the dimethyl amine analog of **47**, **48**, was generated **Scheme 3-3**. Starting from (R)-3-amino-3-(4-methoxyphenyl)propanol **64**, Boc-protection of the free amine gave compound **71**. Then, mesylation of alcohol group with mesylchloride afforded the intermediate **72**. Displacement of the mesylate group with dimethylamine in THF gave tertiary amine intermediate **73**. Acid catalyzed deprotection yielded the free amine **74**, which was coupled to 4-chloro, 6-phenyl, thienopyrimidine to afford the targeted compound **48**. To replace the para methoxy phenyl moiety of **25**, with a less lipophilic, pyridinyl ring, compounds **49** was synthesized following the general procedure for the amination of 4-chloro, 6-phenyl, thienopyrimidine. Next synthetic efforts aimed to diminish the lipophilicity by substitution to phenyl ring at the 6 position of the thienopyrimidine. Pyridyl and pyrazolyl analogs were synthesized as described in

Scheme 3-1. Synthesis of amine 46.

Reagents and Conditions: (i) di-*tert*-butyl decarbonate, Et₃N, DCM, RT, 2 h; (ii) Phthalimide, DIAD, Ph₃P, DCM, RT, 4h; (iii) 4 M HCl in dioxane, DCM, methanol, RT, 4 h; (iv) Et₃N, reflux, 6 h; (v) NH₂NH₂, methanol, RT, 6 h.

Scheme 3-2. Synthesis of the primary amine 47.

Reagents and conditions: (i) Benzyl chloroformate, Et₃N, THF, RT, 14 h; (ii) Phthalimide, DIAD, Ph₃P, DCM, RT, 4h; (iii) NH₂NH₂, methanol, RT, 6 h; (iv) di-*tert*-butyl decarbonate, Et₃N, DCM, RT, 2 h; (v) Pd/C (10 wt%), H₂, methanol, 14 h; (vi) Et₃N, reflux, 6 h; (vii) 4 M HCl in dioxane, DCM, methanol, RT, 4 h.

Scheme 3-3. Synthesis of amine 48.

Reagents and conditions: (i) di-*tert*-butyl dicarbonate, Et₃N, DCM, RT, 2 h; (ii) Methanesulfonyl chloride, Et₃N, DCM, RT, 2 h; (iii) Dimethylamine in ethanol, tetrabutylammonium, 150°C, Microwave, 0.5 h; (iv) 4 M HCl in dioxane, DCM, methanol, RT, 4 h; (v) Ethanol, 150°C, Microwave, 1 h.

Scheme 3-4. Starting from 4-chloro, 6-bromo thienopyrimidine, amination reaction introduced the 4-position side chain and gave the intermediate **76**. Suzuki coupling reaction of **76** with the corresponding aryl boronic acids gave the targeted compounds **50-53**. To remove the 6-phenyl ring and replace it with a methyl motif, **54** was synthesized following the general procedure for the amination of 4-chloro, 6-phenyl thienopyrimidine.

To improve drug clearance of next generation compounds fluorination was explored. Compound **55** was synthesized with a fluorine atom at the para position of the 6-phenyl moiety, as described in **Scheme 3-5**. Starting with fluoro-phenylacetaldehyde **82**, we carried out ring closure to give the fluorophenylthiophene **83** in the presence of ethyl-3-cyanopropanoate **4**. The pyrimidine **84** was formed by heating a solution of compound **83** in excess formamide. Chlorine substituted thienopyrimidine **85** was prepared in the presence of phosphoryl chloride and was coupled with the amine **59** to give the targeted analog **55**.

In a scaffold hopping exercise, to replace the sulfur atom and decrease the lipophilicity and increase the stability of **25**, pyrrolopyrimidine and quinazoline scaffolds were identified.¹³⁴ Compounds **56** and **58** (ClogP: 3.73 and 4.18, respectively) were synthesized as described in **Scheme 3-4**. Compound **57** (ClogP: 4.18) was synthesized as described in **Scheme 3-6**. First, we carried out amination of compound **82** to give the intermediate **83**. Then a Suzuki coupling reaction was prepared with **83** and the boronic acid **77e** to yield targeted compound **57**.

Anti-*H. pylori* Activity of New Analogs

Amine Series. Primary amine compounds **46** and **47** did not show anti-*H. pylori* activity (H.p. IC₅₀ of 2.1 and 2.1 µg/mL respectively, **Table 3-1**) compared to their corresponding alcohols **25** and **45** (H.p. IC₅₀ of 0.006 and 0.08 µg/mL respectively, **Table 3-1**). Fluorination to the side chain phenyl ring produced primary amine **31**, which showed slightly improved activity and therapeutic index (H.p. IC₅₀: 0.54 µg/mL and T. Index: 35.43). The tertiary amine **48** further weakened activity (H.p. IC₅₀: 12.9 µg/mL). To reduce lipophilicity, compound **49** (ClogP = 3.17, **Table 3-1**) was synthesized to replace the para methoxy phenyl motif of **25**, with a pyridinyl ring. Compound **49** showed activity but was cytotoxic, resulting in a low therapeutic index (H.p. IC₅₀: 0.167 µg/mL and T. Index: 92.39, **Table 3-1**).

Heteroaryl Substitution. Pyridinyl substitution at the 6- position theinopyrimidine was well tolerated. **50**, **51** and **52** show good anti-*H. pylori* activities (IC₅₀: 0.112 µg/mL, 0.084 µg/mL and 0.115 µg/mL respectively, **Table 3-2**). However, these compounds are cytotoxic, and demonstrate low therapeutic indices (T. Index: 55.80, 169.17 and 95.91 respectively, **Table 3-2**). Compound **53** with a pyrazole ring at the 6- position, was less potent and more cytotoxic than the corresponding pyridines (H.p. IC₅₀: 0.385 µg/mL, Cytotox IC₅₀: 6.31 µg/mL, **Table 3-2**). The pyridyl and pyrazole decreased the LogP of the series by over one Log unit, suggesting a major increase in solubility.

Scheme 3-4. Synthesis of thienopyrimidine and pyrrolopyrimidine derivatives with modifications at the 6-position.

Reagents and conditions: (i) Ethanol, 150°C, Microwave, 1 h; (ii) Pd(Ph)₄, K₂CO₃, Dioxane, 70°C, 14 h.

Scheme 3-5. Synthesis of fluorophenyl thienopyrimidine 55.

Reagents and conditions: (i) Sulfur, Morpholine, Ethanol, 70°C, Microwave, 20 min, 90%; (ii) Formamide, reflux, 18 h, 80%; (iii) POCl₃, N,N dimethylaniline, reflux, 14 h, 90%; (iv) Ethanol, 150°C, Microwave, 1 h, 93%.

Scheme 3-6. Synthesis of quinazoline 57.

Reagents and conditions: (i) Ethanol, 150°C, Microwave, 1 h; (ii) Pd(Ph)₄, K₂CO₃, Dioxane, 70°C, 14 h.

Table 3-1. Thienopyrimidines with modified moieties at the 4-position for improved solubility and reduced lipophilicity.

Compound	Structure	ClogP	<i>H. pylori</i> IC ₅₀ [μg/mL] SS1 strain	Cytotoxicity IC ₅₀ [μg/mL] FaDu Cells	Therapeutic Index
25		4.23	0.006	200	33,333.33
30		4.31	0.004	12.42	3105
45		4.59	0.077	15.63	202.98
46		4.30	2.1	16.06	7.64
31		4.38	0.538	19.06	35.43

Table 3-1. (Continued).

Compound	Structure	ClogP	<i>H. pylori</i> IC ₅₀ [μg/mL] SS1 strain	Cytotoxicity IC ₅₀ [μg/mL] FaDu Cells	Therapeutic Index
47		4.67	2.09	43.75	20.93
48		5.51	12.92	15.72	1.22
49		3.17	0.167	15.43	92.39

Table 3-2. Thienopyrimidines with modifications at the 4-position.

Compound	Structure	ClogP	<i>H. pylori</i> IC ₅₀ [μg/mL] SS1 strain	Cytotoxicity IC ₅₀ [μg/mL] FaDu Cells	Therapeutic Index
50		2.75	0.112	6.25	55.80
51		2.75	0.084	14.21	169.17
52		2.96	0.115	11.03	95.91
53		2.36	0.385	6.31	16.39
54		3.08	0.123	34.87	283.49
55		4.37	0.016	200	12,500

To reduce lipophilicity, we synthesized compound **54** (ClogP: 3.08, **Table 3-2**) with a methyl group replacing the phenyl ring at the 6-position. Compound **54** shows acceptable activity and a good therapeutic index (H.p. IC₅₀: 0.123 µg/mL, T. Index: 283.49 µg/mL).

Fluorination of Leads. Compound **55**, with a fluorine substituted at the 6-phenyl ring, maintained high potency (H.p. IC₅₀: 0.016 µg/mL, **Table 3-2**) and an excellent therapeutic window. (T. Index: 12,500, **Table 3-2**).

Scaffold Hopping Analogs. The pyrrole-pyrimidine compound **56** (H.p. IC₅₀: 0.065 µg/mL, **Table 3-3**) and the quinazoline compound **57** (H.p. IC₅₀: 0.034 µg/mL, **Table 3-3**) demonstrate high potency and acceptable therapeutic indices (T. Index: 454.31 and 730 respectively, **Table 3-3**). The quinazoline **58** shows reduced potency (H.p. IC₅₀: 0.499 µg/mL, **Table 3-3**), suggesting the phenyl ring is preferred at the 7-position over the 6-position of the quinazoline core.

ADME Properties of New Leads

Since, compounds **30**, **45**, **54**, **55**, **56** and **57** demonstrate good therapeutic indices (> 200), we proceeded to determine the solubility, the human plasma protein binding percentage and the microsomal stability of these compounds (**Table 3-4**). Compared to **25**, compound **30** shows a slightly improved solubility (Sol: 3 µM, **Table 3-4**) and a reduced metabolic stability ($t_{1/2}$ = 0.86 hr, **Table 3-4**). To our surprise, compound **45** show good solubility (Sol: 63.7 µg/mL, **Table 3-4**), a good HPPB% (96.71, **Table 3-4**) and an acceptable metabolic stability ($t_{1/2}$ = 0.99 hr, **Table 3-4**). When comparing compound **25** and **55** (**Table 3-4**), the introduction of a fluorine atom at the para position of the 6-phenyl ring, significantly improve the microsomal stability ($t_{1/2}$: 13.34 hr, **Table 3-4**) but decreased the solubility. The replacement of the phenyl substituent at the 6 position with a methyl motif, as exemplified by compound **54**, significantly improves the solubility (Sol: 69.8 µM, **Table 3-4**) and reduces the plasma protein binding (HPPB%: 95.9, **Table 3-4**). However, this replacement of phenyl with a methyl leads to a major reduction of metabolic stability ($t_{1/2}$: 0.03 hr, **Table 3-4**). The pyrrolopyrimidine analog **56** demonstrates an improved solubility (Sol: 20.1 µM, **Table 3-4**) and a reduced plasma protein binding (HPPB%: 97.9, **Table 3-4**). Although **57** is less bound to plasma protein (HPPB%: 98.1, **Table 3-4**), it does not demonstrate a significant improvement in solubility (Sol: 2.4 µM, **Table 3-4**).

Conclusion

In conclusion, we have introduced targeted chemical modifications to improve the *in vitro* pharmacokinetic properties of the lead compound **25**. Although it demonstrates a high potency (H. p. IC₅₀: 0.006 ug/mL), **25** lacks *in vivo* efficacy due to poor solubility, high protein binding and high lipophilicity. These factors limit the amount of free drug concentration that reaches the *H. pylori* site of infection. Efforts to improve these *in vitro*

Table 3-3. Pyrrolo[2,3-d]pyrimidine and quinazolines synthesized for improved solubility.

Compound	Structure	ClogP	<i>H. pylori</i> IC ₅₀ [μg/mL] SS1 strain	Cytotoxicity IC ₅₀ [μg/mL] FaDu Cells	Therapeutic Index
56		3.73	0.065	29.53	454.31
57		4.18	0.034	24.82	730
58		4.18	0.499	32	64.13

Table 3-4. ADME properties of selected compounds.

Compound	Structure	ClogP	H. p. IC ₅₀ [μg/mL]	Cytotox FaDu Cell [μg/mL]	Solubility [μM]	Human Plasma Binding (%)	Mouse Microsomal Stability t _{1/2} [hr]
25		4.23	0.006	200	1	99.3	3.8
30		4.31	0.004	12.42	0.0	99.78	0.86
45		4.59	0.077	15.63	63.7	96.71	1.78
54		3.08	0.123	34.87	69.8	95.9	0.03
55		4.37	0.016	200	0.5	99.4	13.34
56		3.73	0.065	29.53	20.1	97.9	na

Table 3-4. (Continued).

Compound	Structure	ClogP	H. p. IC ₅₀ [μg/mL]	Cytotox FaDu Cell [μg/mL]	Solubility [μM]	Human Plasma Binding (%)	Mouse Microsomal Stability t _{1/2} [hr]
57		4.18	0.034	24.82	2.4	98.1	na

pharmacokinetic properties revealed compound **56**, which demonstrates an improved solubility and is less bound to plasma protein. The removal of the sulfur greatly improves the solubility and the plasma protein binding. Para-fluoro substitution at the 6-phenyl group significantly improves the microsomal stability of the series. The pyrrolopyrimidine scaffold is a promising core in the optimization of the ADME properties, to discover a candidate for *in vivo* efficacy studies in the *H. pylori* murine model.

CHAPTER 4. CONCLUSION

H. pylori is the causative agent of gastritis, peptic ulcers and gastric cancer. The recommended therapy for *H. pylori* infections is the triple therapy which consists of a PPI, and two antibiotics including clarithromycin and amoxicillin. The rate of successful treatment with the triple therapy has dropped below 80%, due to the global rise of resistance against these antibiotics. The World Health Organization lists *H. pylori* as a high priority drug-resistant bacterium. A narrow-spectrum drug is needed to address the drug resistance challenge and preserve these broad-spectrum antibiotics for the treatment of other types of infections. Advances in whole genome sequencing provide an opportunity to discover new chemical matter. Recent transposon mutagenesis studies have revealed *H. pylori* specific genes that enable the pathogen to grow in the stomach. The existence of these genes suggests that there are novel and unique targets that can be explored for the development of novel therapies against *H. pylori* infections. Phenotypic screening for selective compounds against *H. pylori* is a promising approach to unveil these targets.

In an effort to discover a new chemical matter, we conducted a high-throughput screen of 219,197 compounds. Positive hits were counter-screened against a number of gut commensals, to eliminate promiscuous compounds. Two thienopyrimidines emerged from this screen and were explored through the synthesis of 29 analogs and the structure activity relationship (SAR) studies that merged elements from both hits. The SAR revealed several potent compounds, in low $\mu\text{g/mL}$ -range of activity, including compound **25** (H.p. IC_{50} : 0.006 $\mu\text{g/mL}$). Mode of action studies that involved the generation of mutants, revealed NuoD, a subunit of *H. pylori*'s respiratory Complex I, as the putative target of the thienopyrimidine. Complex I is an enzyme that catalyzes the first step of oxidative phosphorylation.

Targeting oxidative phosphorylation has been proven to be beneficial in the clinic, as exemplified by metformin, a drug that is used for the treatment of diabetes. The inhibition of oxidative phosphorylation is becoming an important approach in the development of therapies for the treatment of AML and solid tumors. Additionally, NuoD has been found to be essential for *H. pylori* but not for most of other bacteria including *E. coli*. These realizations suggest that the thienopyrimidines are a promising series of compounds, worth further optimization to fulfill the eradication of *H. pylori* in the *in vivo* murine model. The lead thienopyrimidine compound **25** eradicates *H. pylori* in the *ex vivo* model but lacks *in vivo* efficacy, suggesting the optimization of pharmacokinetic properties of the lead compound **25** is required.

To address the limitations of **25**, we have synthesized 16 additional compounds. At 50 mg/kg, compound **25** shows good exposure (AUC: 31907.71 hr*ng/mL) with a high volume of distribution (V_{ss} : 6.28 L/Kg), suggesting that **25** (Clogp : 4.23) is too lipophilic and is being trapped in fatty tissues. **25** also shows poor solubility (Sol: 0.1 μM) and high plasma protein binding (HPPB: 99.3 %), suggesting not enough of free drug concentration is able to reach the site of infection, in the mucus layer of the

stomach. New compounds include amine derivatives of **25** and other lead compounds such as **30** and **45**, to improve the solubility of the series. Scaffold hopping exercise that aimed to remove the lipophilic sulfur atom, revealed pyrrolopyrimidine and quinazoline scaffolds. The pyrrolopyrimidine analog **56** is less lipophilic (ClogP: 3.73), demonstrates an improved solubility (Sol: 20.1 μ M) and is less bound to plasma protein (HPPB: 97.9 %) compared to lead compound **25**. Compound **56** is a promising compound, in the lead optimization efforts toward the *in vivo* proof of concept studies in the *H. pylori* murine model. 6-pyridinyl substituted derivative of pyrrolopyrimidine **56** will likely lead to improved solubility and decreased plasma protein binding. Fluorination of the 6-aryl ring will prolong the half-life of drug *in vivo*. Substitution of the (R)-3-amino-3-(4-methoxyphenyl) propanol motif, identified with compound **45**, at the 4-position of the pyrrolopyrimidine core could lead to a series with improved solubility and low plasma protein binding.

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APPENDIX.

Table A-1. Inhibition profile of thienopyrimidines against *H. pylori* ATCC 43504 strain, SS1 strain and SS1 mutant strain.

Compound		<i>H. pylori</i> IC ₅₀ (µg/ml)				Cytotoxicity IC ₅₀ (µg/ml) HepG2 Cells
		ATCC 43504 Strain	SS1 Strain	SS1 nuoD A402P	Ratio	
1		0.44 ± 0.19	0.46 ± 0.07	15.42 ± 11.51	33.58	42.68 ± 14.00
8		>10 ± 0.00	1.85 ± 1.48	15.48 ± 12.61	8.38	36.52 ± 9.44
9		1.53 ± 0.43	0.55 ± 0.22	20.19 ± 12.39	36.96	81.55 ± 26.07
10		0.01 ± 0.007	0.05 ± 0.09	3.04 ± 3.38	55.00	46.83 ± 4.08
11		0.34 ± 0.24	0.13 ± 0.07	5.50 ± 2.79	43.46	51.05 ± 4.96
12		1.55 ± 0.21	0.98 ± 0.71	5.59 ± 2.92	5.73	103.67 ± 23.29
13		0.05 ± 0.007	0.01 ± 0.007	3.35 ± 3.28	267.39	96.49 ± 9.64
14		0.41 ± 0.24	0.16 ± 0.09	5.42 ± 2.69	34.81	42.64 ± 10.81
15		0.72 ± 0.33	0.18 ± 0.12	11.04 ± 13.92	61.18	>200 ± 2.27
16		0.03 ± 0.006	0.02 ± 0.00	2.59 ± 3.07	162.38	43.56 ± 5.42
17		0.31 ± 0.21	0.08 ± 0.05	4.89 ± 3.43	55.68	26.77 ± 0.61
18		1.09 ± 0.12	0.24 ± 0.05	14.28 ± 11.30	58.40	33.58 ± 9.96

Table A-1. (Continued).

Compound		<i>H. pylori</i> IC ₅₀ (µg/ml)				Cytotoxicity IC ₅₀ (µg/ml) HepG2 Cells
		ATCC 43504 Strain	SS1 Strain	SS1 nuoD A402P	Ratio	
19		0.08 ± 0.06	0.02 ± 0.002	0.29 ± 0.33	17.68	>200 ± 0.00
20		0.03 ± 0.004	0.22 ± 0.17	3.34 ± 3.41	15.36	135.01 ± 40.25
21		0.92 ± 0.30	0.48 ± 0.32	1.27 ± 1.87	2.63	29.26 ± 3.34
22		1.97 ± 0.71	1.08 ± 0.47	4.4 ± 2.98	4.07	31.86 ± 3.38
23		0.57 ± 0.08	0.24 ± 0.17	5.48 ± 2.83	23.13	12.45 ± 1.70
24		0.06 ± 0.03	0.09 ± 0.03	3.53 ± 0.29	41.12	6.51 ± 11.515
25		0.004 ± 0.000	0.004 ± 0.000	0.1 ± 0.09	18.41	>200 ± 0.00
26		0.004 ± 0.002	0.004 ± 0.000	0.06 ± 0.05	15.59	18.76 ± 3.64
27		0.02 ± 0.003	0.05 ± 0.045	1.75 ± 0.71	45.55	28.22 ± 0.54
28		0.02 ± 0.003	0.01 ± 0.007	0.44 ± 0.31	39.11	<6.25 ± 0.00

Table A-1. (Continued).

Compound		<i>H. pylori</i> IC ₅₀ (µg/ml)				Cytotoxicity IC ₅₀ (µg/ml) HepG2 Cells
		ATCC 43504 Strain	SS1 Strain	SS1 nuoD A402P	Ratio	
29		0.009 ± 0.003	0.004 ± 0.001	0.22 ± 0.17	58.03	7.59 ± 1.74
30		0.007 ± 0.002	0.004 ± 0.000	0.19 ± 0.16	49.01	10.71 ± 2.94
31		1.19 ± 0.27	0.54 ± 0.36	6.92 ± 1.075	12.87	<6.25 ± 0.00

Table A-2. Inhibition profile of thienopyrimidines with modifications at the 5 and 6 positions against *H. pylori* ATCC 43504 strain, SS1 strain and SS1 mutant strain.

Compound			<i>H. pylori</i> IC ₅₀ (μg/ml)				Cytotoxicity IC ₅₀ (μg/ml) HepG2 Cells
	R ₁	R ₂	ATCC 43504 Strain	SS1 Strain	SS1 nuoD A402P	Ratio	
37	CH ₃	CH ₃	1.77 ± 0.05	0.25 ± 0.18	5.05 ± 3.26	19.89	<6.25 ± 0.00
38	CH ₃		0.12 ± 0.007	0.02 ± 0.001	3.71 ± 2.87	222.93	20.64 ± 6.00
39		CH ₃	1.51 ± 1.49	10.24 ± 13.58	10.51 ± 12.95	1.03	32.93 ± 1.92
40		H	1.51 ± 0.27	0.215 ± 0.16	0.44 ± 0.35	2.06	27.86 ± 9.41

Table A-3. ADME of the lead compound 25.

Compound	MW	ClogP	Solubility [μM]	PAMPA ^a Avg Pe (10 ⁻⁶ cm/s)	Mouse Plasma Binding (%)	Microsomal Stability (Mouse) t _{1/2} [hr]
25	377	4.23	1 ± 0.2	239.5 ± 180.5	99.3 ± 0.1	3.8 ± 0.37

^a The PAMPA assay was carried out at physiological (pH = 7.4).

Table A-4. Evaluation of *in vivo* pharmacokinetic properties of the lead compound 25.

Compound	Route	Dose [mg/kg]	T _{max} (hr)	C _{max} (ng/mL)	AUC _{last} (hr*ng/mL)	T _{1/2} (hr)	CL (mL/min/kg)	V _{ss} (L/kg)
25	i.p.	50	2.00	2550.25	31 907.71	2.79	26.05	6.28
	PO	10	0.25	589.84	674.42	1.52	-	-

Figure A-1. *In vivo* efficacy of compound 25 in the *H. pylori* infected mouse model. C57BL16 mice were infected with a *H. pylori* SS1 strain suspension. Then, mice were treated with metronidazole as control, and the following doses of compound **25**, twice per day: 50 mg/kg (OG), 50 mg/kg (IP) and 25 mg/kg (IP).

VITA

Alex K. Mugengana was born in Mwanza, Tanzania in 1988. He received a bachelor's degree in Chemistry from Philander Smith College in Little Rock, Arkansas. After he graduated from college, he trained as an intern in the laboratory of Dr. William Tolleson at the National Center for Toxicological Research/ Food and Drug Administration (NCTR/FDA) in Jefferson, Arkansas. In 2013, he joined the pharmaceutical sciences graduate program at UTHSC to pursue his doctoral studies. In the following year, he served as vice-president in the Graduate Student Executive Committee. He is a recipient of the Honor Medal from the Office of International Program/ FDA and was recognized at the Black Student Association (BSA) Annual Awards Banquet as the 2016 College of Graduate Health Science (CGHS) Outstanding Student. In December of 2019, he will graduate with a Doctor of Philosophy degree.