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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Next Generation 5-Nitroimidazole Compounds Show Potential as New Therapeutic Alternatives to
Metronidazole in *Helicobacter pylori* Infection

A thesis submitted in partial satisfaction of the requirement for the degree of Master of Science

in

Biology

by

Ricardo Lozano

Committee in charge:

Professor Lars Eckmann, Chair
Professor James Golden, Co-Chair
Professor Milton Saier

2013

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Co-Chair

Chair

University of California, San Diego

2013

DEDICATION

Yo dedico este tesis a mi mamá, papá, y Fufis
por su amor y apoyo.

EPIGRAPH

“Gool....I meant to say cool and then I started to say great.”
Cady Haren

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Finally, I want to thank my friends and family for all their encouragement and support.

ABSTRACT OF THE THESIS

Next Generation 5-Nitroimidazole Compounds Show Potential as New Therapeutic Alternatives to Metronidazole in *Helicobacter pylori*

by

Ricardo Lozano

Master of Science in Biology

University of California, San Diego, 2013

Professor Lars Eckmann, Chair
Professor James Golden, Co-Chair

5-Nitroimidazole (5-NI) compounds are used to treat a variety of bacterial and parasitic infections. Metronidazole (Mz), a 5-NI drug, is a safe and affordable drug that has been used for nearly half a century to treat infections caused by intestinal pathogens. When combined with other medications Mz has successfully been used to treat *H. pylori* infections. *H. pylori* is a microaerophilic bacteria that causes a range of gastrointestinal diseases. While Mz is active against *H. pylori*, the treatment failure rate of Mz centered therapy is increasing (Peter J. Jenks 2002). Others have taken advantage of the ability to synthesize structurally diverse 5-NI compounds and have found that some of the newly derived compounds are highly potent compared to Mz (Jacqueline A. Upcroft 2006). We asked if 5-Ni compounds, apart from Mz, could reduce the growth rate of *H. pylori* and potentially function as new antibiotics. We show that 5-NI compounds are active against multiple strains of *H. pylori*. Also, we found four compounds that

are able to overcome Mz-resistance. Finally, we propose a new method for gaining a better understanding of Mz resistance mechanisms using a strain's compound activity profile coupled with phylogenetic tools.

INTRODUCTION

Overview

Helicobacter pylori is a microaerophilic, helical shaped, gram-negative bacterium that inhabits the gastric mucosa of half the world's population (Suerbaum and Michetti 2002). It is most commonly spread from person-to-person, however, waterborne transmission is thought to be another route to infection (Brown 2000). *H. pylori* is able to survive in the stomach by utilizing an enzyme called urease. Urease works by catalyzing the hydrolysis of urea to yield ammonium, which helps neutralize the acidic environment the bacteria inhabits. The enzyme helps *H. pylori* survive, yet it also functions as an immunogen and elicits an immune response (Molbey HTL 2001). After the isolation of *H. pylori* by Marshall and Warren in 1982, the bacterium was connected to a range of gastrointestinal diseases. However, not everyone who is infected suffers from peptic ulcers or gastric malignancy associated with infection (Rothenbacher and Brenner 2003). Susceptibility to *H. pylori* infection varies between geographic area and socioeconomic class. While prevalence of childhood infection can range from 40-60% in developing countries, less than 10% of children in developed countries are infected. In the United States, for example, infection is low among white and economically wealthy Americans while minority populations have higher frequency of infection (Malaty 2007).

Treatment for *H. pylori* Infection

H. pylori infection is verified by serological examination, urease-breath test, or biopsy test. Once infection is confirmed a three-drug treatment regimen is prescribed. This "triple therapy" usually consists of a proton-pump inhibitor, clarithromycin, and amoxicillin or metronidazole (P Malfetheiner 2007). However, because of an increase in antibiotic resistant strains of *H. pylori* there are alternative treatment regimens (Seng-Kee Chuah 2011). Apart from its role as an antibiotic used in the "triple therapy" regimen metronidazole (Mz) also plays an important role in the treatment of many different bacterial and parasitic infections such as trichomoniasis, amoebiasis, and giardiasis (Löfmark, Edlund et al. 2010). The wide applicability,

low cost, and favorable pharmacological properties of Mz make it a World Health Organization essential drug, particularly in countries without access to a wide range of antibiotics.

Mz Mechanism of Action

Metronidazole, 2-(2-methyl-5-nitro-1H-imidazole-1-yl) ethanol, is a prodrug that is activated through the reduction of its nitro group (Edwards 1993). Mz enters the cell through passive diffusion. Reduction of the nitro group converts Mz into a radical anion, which decreases the concentration of Mz in the cell and creates a concentration gradient for Mz (Jorgensen, Manos et al. 1998). Mz is able to selectively target anaerobic and microaerophilic organisms because of its low reduction potential. In order for Mz to exert its killing effects an organism must be able to reduce the prodrug with reactions that have a redox potential more negative than the reduction potential of Mz. Humans are not affected by Mz because the redox couple with the lowest potential in humans, the NADP/NADPH couple (-324 mV), has a higher redox potential than Mz (-415 mV). Studies of Mz-resistant strains of *H. pylori* have led to the discovery of two nitroreductases involved in Mz activation, oxygen-insensitive NADPH nitroreductase (rdxA) and NADPH-flavin-oxioreductase (FrxA) (Jin-Yong Jeong 2001). While the mechanism of Mz reduction in obligate anaerobes is well understood, the increased oxygen tension experienced by microaerophiles complicates the situation for *H. pylori*. Oxygen is a better electron acceptor than Mz. Therefore, molecular oxygen can remove the electron from the reduced nitro group on Mz and regenerate the Mz prodrug. Regeneration of Mz into its inactive prodrug form by oxygen is known as “futile cycling” (George L. Mendez 2002). One product of futile cycling, the hydroxyl radical, is known to damage DNA. However, not this, nor other metabolites of futile cycling, are responsible for the killing effect of Mz (Jorgensen, Manos et al. 1998). Whether other mechanisms are responsible for Mz activation in *H. pylori* remains unknown.

Mz Resistance in *H. pylori*

Failure to clear *H. pylori* infection with Mz-based therapy varies between different regions. While the rate of Mz treatment failure is 44% in North and South America, it is 43% in Africa, 37% in Asia, and only 17% in Europe (Wenning Wu 2012). Treatment failure is indicative

of infection with an Mz resistant strain of *H. pylori*. A strain is considered resistant if it can grow *in vitro* in at least 8 µg/ml of Mz. Possible mechanisms for resistance to Mz studied so far include: alteration in drug efflux/influx, enhanced activity of DNA repair enzymes, and increased oxygen-scavenging ability (George L. Mendez 2002). However, the principle mechanism of Mz resistance in *H. pylori* appears to involve decreased drug activation, specifically attenuation or inactivation of the nitroreductases RdxA and FrxA. Currently, two theories exist regarding the role of rdxA and frxA in Mz-resistance. One theory suggests inactivation of either rdxA or frxA/rdxB can initiate Mz-resistance. This theory attributes the range of Mz-resistance phenotypes to the multiple combinations of nitroreductase inactivation (Dong-Hyeon Kwon 2000). The other theory proposes there are two types of Mz-sensitive *H. pylori*. Type I, requires only inactivation of rdxA to become resistant while type II requires inactivation of both rdxA and frxA to confer resistance. However, Mz resistance cannot be initiated by inactivation of frxA alone (Jin-Yong Jeong 2000).

Application of Structurally Modified 5-NI Compounds

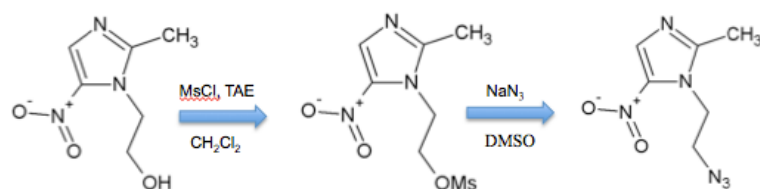
The 5-NI core in nitro drugs can be modified with a range of different substituents to create novel 5-NI compounds. Previous experiments have demonstrated that structurally diverse 5-NI compounds can be more potent than Mz in Mz-sensitive and Mz-resistant parasites (Jacqueline A. Upcroft 2006). However, the effectiveness of modified 5-NI compounds remained to be examined in bacteria, such as *H. pylori*. Therefore, this study had the goal to identify new 5-NI compounds that can overcome resistance in *H. pylori* using a library of more than 600 novel and structurally diverse 5-NI compounds.

RESULTS

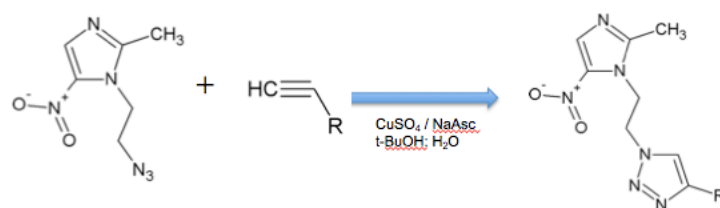
New Generation 5-NI Compounds Active Against SS1

The potential of using structurally diverse 5-NI compounds as antibiotics has been proven in parasites such as *Giardia* and *Trichomonas* (Jacqueline A. Upcroft 2006). However, whether a modified 5-NI compound would be potent against the microaerophilic bacterium *H. pylori* remained to be established. Utilizing a modular synthetic approach, 660 new unique 5-NI compounds were synthesized. Each 5-NI compound is composed of two units: the core and the alkyne. The core unit contains the nitro substituent and an azide subunit. There are six different core unit configurations. There are 110 different alkyne substituent units. The azide substituent of the core reacts with the alkyne through an azide-alkyne Huisgen cycloaddition reaction thus creating 660 unique 5-NI compounds (figure 1).

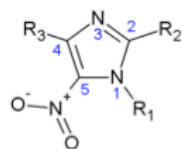
A.



B.



C.



Core	R ₁	R ₂	R ₃
1		CH ₃	H
2	CH ₃		H
3	CH ₃		H
4	CH ₃	CH ₃	
5			H
6			H

Figure 1. 5-NI Compound Composition

(A) Creation of core A

(B) Click reaction

(C) Different core configurations

We began the screening experiments with *H. pylori* Sydney Strain 1 because it is a representative Mz-sensitive strain of *H. pylori* with a sequenced genome, the ability to infect mice, and Mz-resistant isolates of SS1 can easily be created *in vitro* (Jin-Young Jeong 2000). If the new 5-NI compounds have any hope of becoming antibiotics in the future they need to be effective in an Mz-sensitive strain such as SS1. We used a compound's pEC50 value to express its potency. Each compound has a unique EC50 or, half maximal effective concentration, which represents the concentration it takes for a compound to reduce growth and survival by 50%. The negative log value of the concentration required to reduce growth by 50% is the pEC50. In SS1 the pEC50 for Mz is 6.01 ± 0.01 , 49% of the 649 5-NI compounds tested were more potent than Mz and 0.46% were "highly potent" or at least ten times more potent than Mz (figure 2). These results suggest that 5-NI compounds have the potential to serve as antibiotics against *H. pylori* infection.

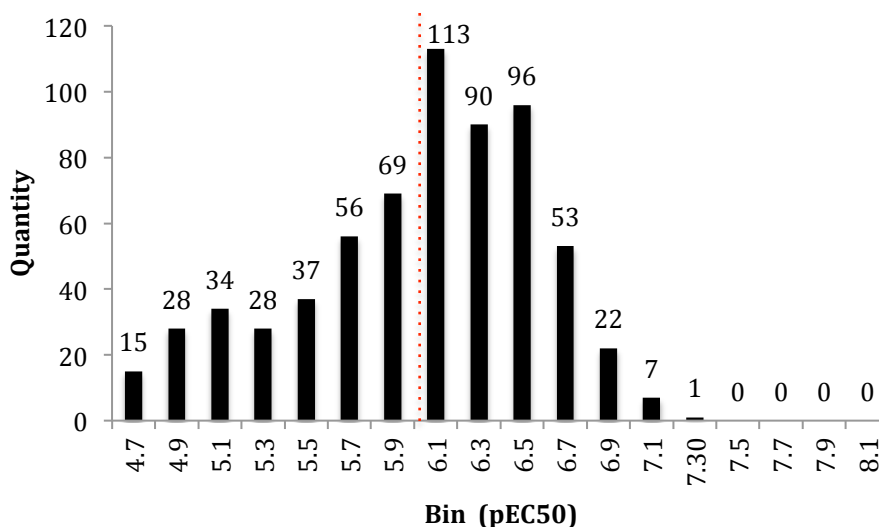


Figure 2. Histogram of Compound Distribution for SS1

The number above each bar represents the amount of compounds that have a pEC50 equal to the number corresponding to the bin and greater to the pEC50 of the bin on the left. The bars to the right of the red line represent compounds with more potency than Mz.

Screening of South African Strain Expands Scope of 5-NI Utility

In order for a new drug to be successful at treating a bacterium with the genetic diversity seen in *H. pylori* it must have the potential to work against more than one strain. We knew the 5-NI compounds were active against SS1. Next, we asked how many of the compounds were superior to Mz in a different strain? In order to answer this questions we screened the *H. pylori* strain CS22, isolated in South Africa, against the 5-NI compound library. The pEC50 of MZ in CS22 was 4.90 ± 0.05 , signifying that CS22 is about ten times more resistant to MZ than SS1. 56% of the compounds screened were more potent than Mz in CS22 and 9.5% of compounds were at least ten times as potent as Mz (figure 3).

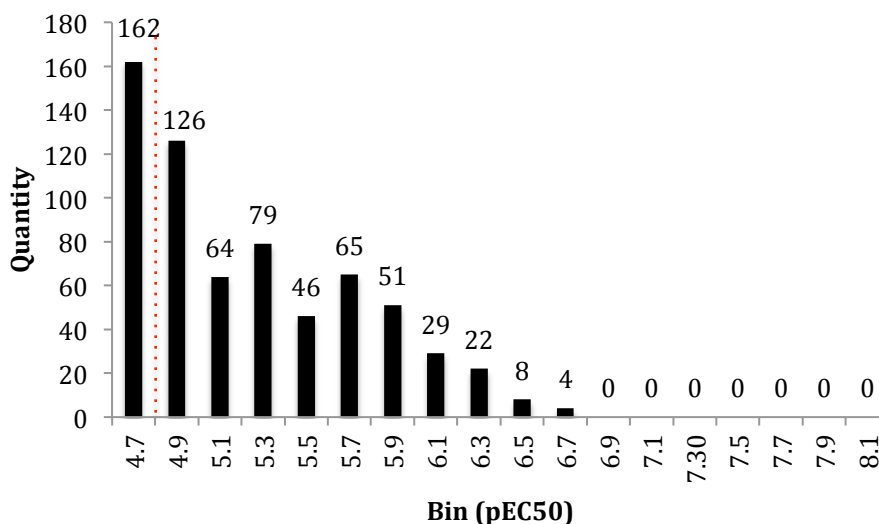


Figure 3. Histogram of Compound Distribution for CS22

The number above each bar represents the amount of compounds that have a pEC50 equal to the number corresponding to the bin and greater to the pEC50 of the bin on the left. The bars to the right of the red line represent compounds with more potency than Mz.

In general, the 5-NI compounds are more potent than Mz in CS22 compared to SS1. This can be demonstrated by comparing the number of compounds with potency values at least ten times greater than Mz in each strain, 9.5% in CS22, and 0.46% in SS1. The compound activity histograms showed a shift of the curve to the left for CS22 compared to SS1, which might be caused by an increase in Mz-resistance in CS22 compared to SS1. However, that shift does not

show how potency of the individual compounds compared between the strains. Were the growth inhibiting effects of the compounds similar between the two strains? In order to determine if the overall shift was a result of a proportional decrease or if there was no relation in compound activity between SS1 and CS22 the screening data for the two strains was plotted on a pairwise scatter graph. To interpret the scatter graph data the coefficient of determination, R^2 , was calculated and found to be 0.36 (figure4).

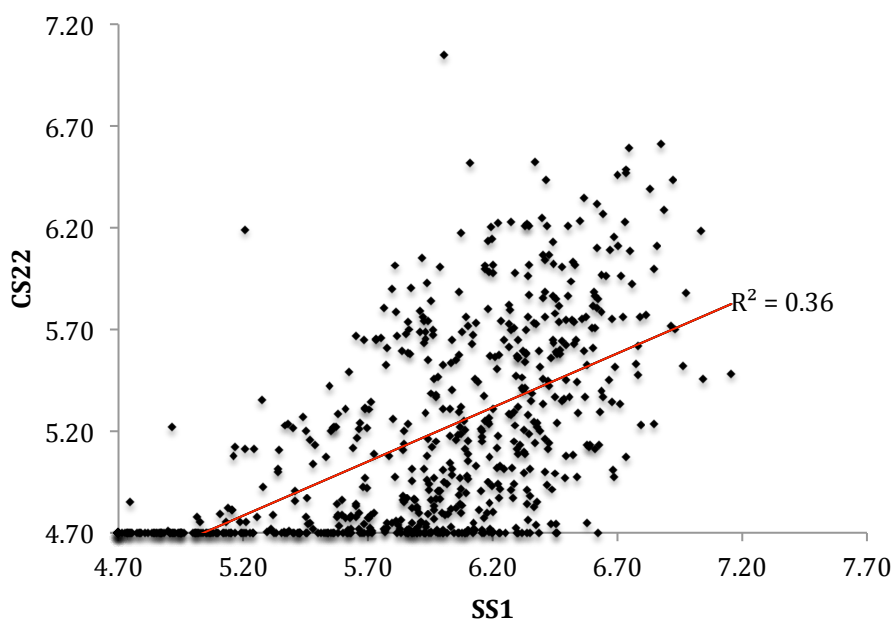


Figure 4. Compound Activity Landscape of CS22 and SS1

Each point represents one 5-NI compound. X- axis corresponds to the pEC50 of the compound in SS1 y-axis corresponds to the pEC50 of the compound in CS22. The trend line is in red.

Since R^2 analysis gave us ambiguous results we decided to calculate the correlation coefficient of the compound activity profiles between the two strains. Also, while R^2 represents the proportion of variability in a data set, it is not representative of the direction or closeness of the linear relationship between the two strains. In order to interpret the degree of similarity in a more quantitative fashion the correlation coefficient, R , was determined. R was 0.50 with a probability that the R value occurred by chance below 0.05%, indicating that the R measurement is highly significant. An R -value of 0.50 is generally considered to be at the cutoff between moderate and

high correlation. Consequently, these results indicate that there is a moderate to high degree of correlation of compound activity between both strains. These results show that 5-NI compound activities against *H. pylori* are not strain specific. This is important because it indicates that the new compounds have the potential to be active any typical strain that might infect patients.

5-NI compounds Can Overcome Mz-Resistance

Evidence of 5-NI compound activity against geographically diverse Mz-sensitive strains prompted questions regarding the ability of the new compounds to kill Mz-resistant strains. We have shown that the 5-NI compounds are more potent than Mz in sensitive strains, but are any of the new compounds able to overcome Mz-resistance in *H. pylori*? If a compound was more potent than Mz in Mz-resistant strains of *H. pylori* we considered it able to overcome the Mz-resistance mechanisms of the cell. To search for 5-NI compounds with the ability to circumvent Mz-resistance mechanisms we created new four strains of *H. pylori* with a range of Mz-resistance phenotypes. Three strains, SS1.05F, 1.5R41B, and 0.5R3241.25A, were isolated in the laboratory after growth on agar plates infused with Mz at different concentrations. In addition, a genetically modified strain of *H. pylori* SS1, RdxA:FrxA, was also screened (Jin-Yong Jeong 2001). RdxA:FrxA has two inactive nitroreductases that are normally responsible for activating Mz, which are active in SS1. In SS1.05F Mz has a pEC₅₀ of 5.72 ± 0.01 , making it the least resistant of the Mz-resistant lines. 63% of compounds screened in SS1.05F were more potent than Mz. 3.4% of compounds were at least ten times more potent than Mz and one compound was 100 times more potent than Mz in SS1.05F (figure 5a). The observation that a greater number of 5-NI compounds are more potent than Mz in SS1.05F compared to its parental strain SS1 might be because Mz-resistance mechanisms affect Mz more than they affect the other 5-NI compounds. The pEC₅₀ for Mz is 4.15 ± 0.13 in strain 1.5R41B. In this strain only 3% of the 658 compounds screened produced a measurable pEC₅₀ and one compound was ten times more potent than Mz (figure 5b).

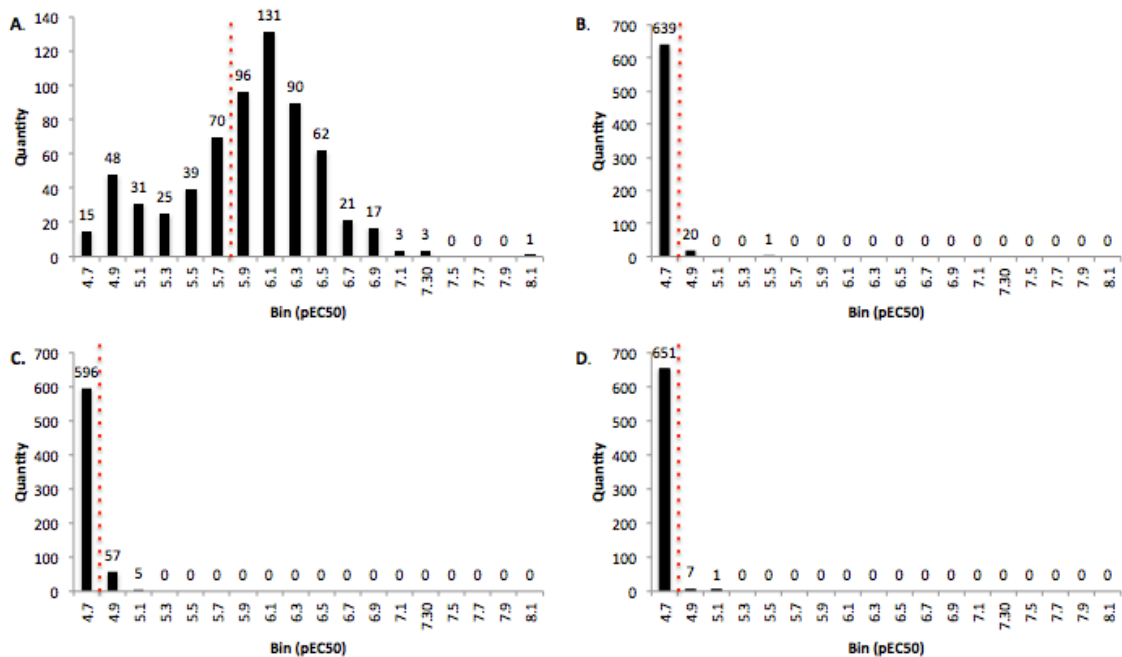


Figure 5. Histogram of Compound Distribution for Mz-Resistant Strains

(A) Strain SS1.05F. The number above each bar represents the amount of compounds that have a pEC50 equal to the number corresponding to the bin and greater to the pEC50 of the bin on the left. The bars to the right of the red line represent compounds with more potency than Mz.

(B) Strain 1.5R41B

(C) Strain 0.5R341.25A

(D) Strain RdxA:FrxA

Strain 0.5R3241.25A has a pEC50 of value of 3.92 ± 0.06 for Mz, making it the most Mz-resistant laboratory derived strain screened. 8.6% out of the 656 compounds screened produced compounds with a pEC50 value within the sensitivity of the screening procedure, more than two times as many compounds than 1.5R41B (figure 5c). Three compounds were ten times more potent than Mz in 0.5R3241.25A. This was surprising because 0.5R3241.25A is resistant to a higher concentration of Mz than strain 1.5R41B. The pEC50 of Mz in strain RdxA:FrxA was the smallest, 3.77 ± 0.05 . This strain is 100 times more resistant to Mz than SS1. Because the strain was resistance to high levels of Mz we conducted a qualitative growth rate experiment before we measured the potency of individual compounds. We only measured the potency of compounds

that inhibited the growth rate of *H. pylori* by at least 25%. 39 compounds of the 659 compounds we screened reduced the growth rate by at least 25%. We found that only eight compounds of the 39 had measurable EC50 values. All eight of those compounds were more potent than Mz in RdxA:FrxA and half were at least ten times more potent than Mz (figure 5d). These results show that some of the compounds in the new 5-NI compound library are able to overcome Mz-resistance in each strain tested. While the quantity of compounds with measurable EC50 values in each strain decreased with increasing Mz-resistance, the same trend was not observed in compounds with ten times more potency than Mz. Overall, these results prove that a number of 5-NI compounds have the ability to overcome Mz-resistance.

Most Potent 5-NI Compounds in Mz-Resistant Strains Also Highly Potent in Mz-Sensitive Strain

After screening four Mz-Resistant strains we noticed there was a clear drop-off in the number of compounds with measurable EC50 values between Mz-resistant strains SS1.05F and RdxA:FrxA. At the least Mz-resistant end of the Mz-resistance spectrum strain SS1.05F was responsive to 98% of compounds. While only 1.2% of compounds produced a measurable effect in the RdxA:FrxA, the most Mz-resistant strain tested. We asked if it is possible to use a Mz-resistant strain with high levels of Mz-resistance to screen for compounds that would also be potent in Mz-sensitive strains? Using a graph comparing compound activity between SS1 and CS22 we plotted the top eight compounds from SS1.05F and RdxA:FrxA and compared the location of the top compounds between the Mz-resistant and Mz-sensitive strains. We split the graphs into four sections based on the pEC50 value that corresponded to the top 25% of the compounds in each of the Mz-sensitive strain. The top eight compounds in RdxA:FrxA were not identical to the top eight compounds in the sensitive strains but, six out of eight were grouped in the top quadrant representing highly potent compounds (figure 6).

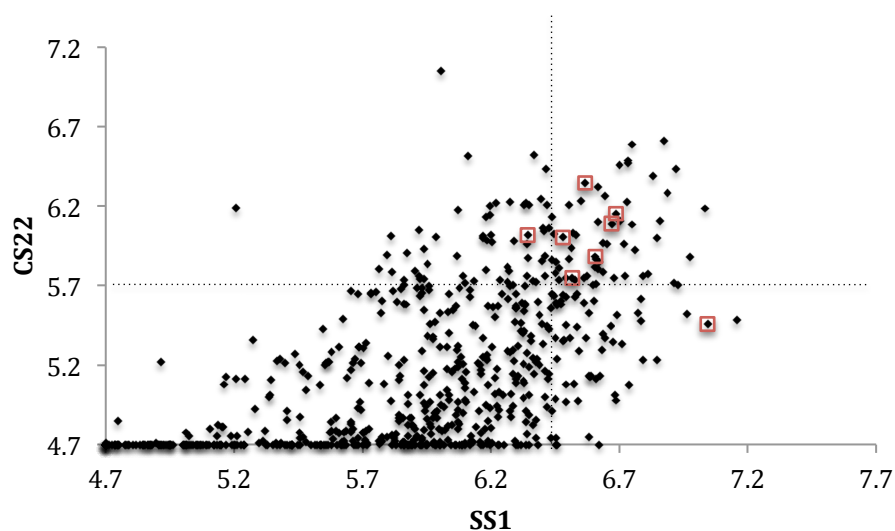


Figure 6. Comparison of 8 Most Potent Compounds From RdxA:FrxA in SS1 and CS22

Black diamond represents individual 5-NI compound, red square represents most potent compound from RdxA:FrxA. Quadrant in top right represents 25% most potent compounds from CS22 and SS1.

The top eight compounds between SS1.05F and the two sensitive strains were also not identical. Unlike the compounds from RdxA:FrxA, the top compounds in SS1.05F were not as tightly clustered in the top quadrant. Only four of the eight most potent compounds in SS1.05F were clustered in the top quadrant (figure 7).

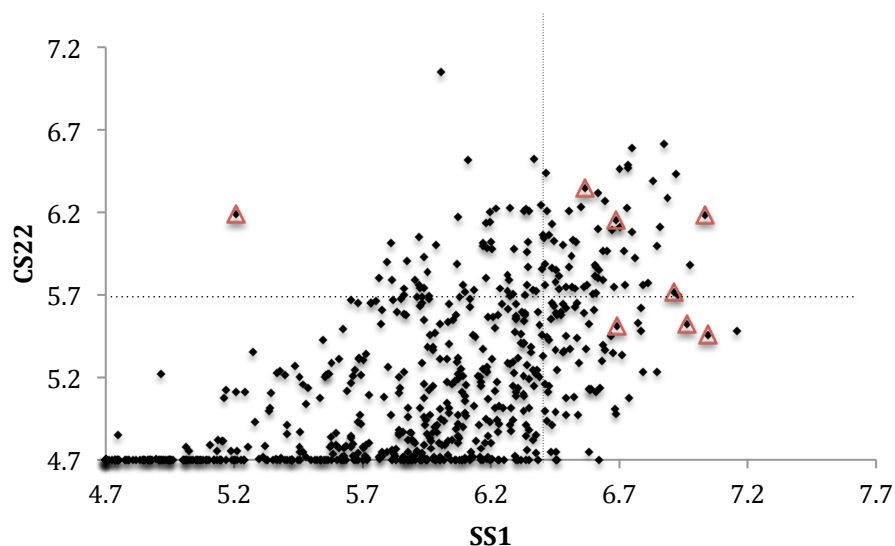


Figure 7. Comparison of 8 Most Potent Compounds From SS1.05F in SS1 and CS22

Black diamond represents individual 5-NI compound, red square represents most potent compound from SS1.05F. Quadrant in top right represents 25% most potent compounds from CS22 and SS1.

Compared to SS1.05F, RdxA:FrxA, the more Mz-resistant strain, is more predicative of compound potency in Mz-sensitive lines. While 75% of compounds in RdxA:FrxA are grouped in the quadrant representing highly potent compounds in both strains the same is true for 50% of the compounds in strain SS1.05F. These results indicate that the most potent compounds in Mz-resistant strains are generally the same compounds that are highly potent in Mz-sensitive strains. We also found that as a strain becomes more Mz-resistant, the most potent compounds in that strain are more likely to be highly potent compounds in Mz-sensitive strains.

Four Broad-Strain Compounds Represent New Drug Candidates

After screening four different Mz-resistant strains of *H. pylori* against the 5-NI compound library it was clear that compounds with higher potency than Mz existed in each strain. Yet, were there any compounds that were more potent than Mz in not just one, but across all Mz-resistant strains of *H. pylori* screened? To figure this out we analyzed the data from the four screening experiments to sort out compounds that were more potent than Mz, or that produced measurable EC50 values. We were left with four compounds that were more potent than Mz in all four

resistant strains and both of the Mz-sensitive strains; they represent the best candidate compounds for future analysis and development (table 1).

Table 1. pEC50 Values of the Four Broad-Strain Compounds and Mz

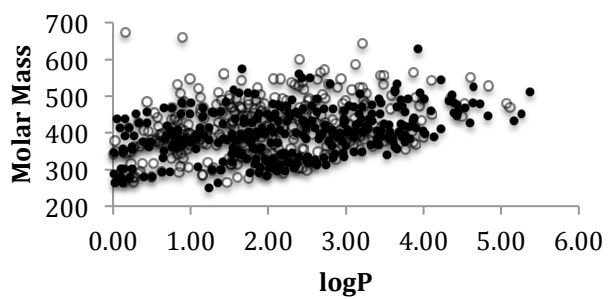
Compound	SS1	CS22	SS1.05F	1.5R41B	0.5R3241.25A	rdxAfrxA
JAR-3-210i	6.48	6.00	6.79	4.86	4.90	4.85
JAR-3-214g	6.52	5.75	6.47	4.83	4.75	4.77
JAR-3-215d	6.61	5.88	6.38	4.77	4.72	4.74
JAR-3-215h	6.34	6.02	6.39	4.87	4.79	4.73
Metronidazole	6.01	4.90	5.72	4.15	3.95	3.77

The discovery of these four broadly potent active compounds represents an important step in our search for drugs that are able to overcome Mz-resistance. Out of those four compounds, Jar-3-210i, was more than ten times as potent than Mz in most of the strains.

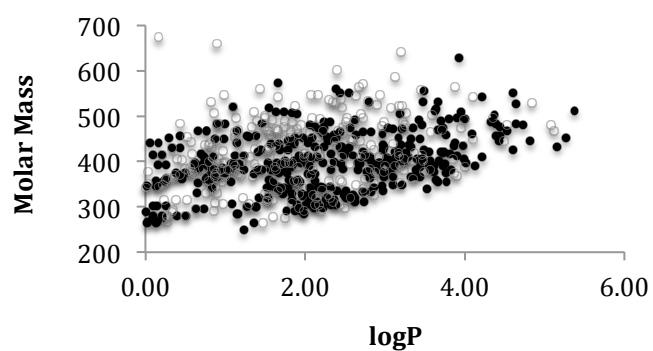
Relationship Between Molar Mass/LogP and Compound Potency

The compound library contains a diverse array of 5-NI compounds. Investigating the physical properties of active compounds could help streamline the design of 5-NI antibiotics in the future. In this section a compound is considered “active” if it is more potent than Mz. We studied two physicochemical properties of the 5-NI compounds: molar mass and logP. The logP, or partition coefficient, is a measure of the hydrophilicity or hydrophobicity of a compound and, therefore, is an important property that affects the pharmacokinetics and *in vivo* activity of a drug. We asked if active compounds share similar logP and molar mass values, is there a range of value for either property that is both sufficient and required to predict compound activity? To answer our question we plotted the logP and molar mass of each compound and distinguished the active compounds from those that were inactive (figure 8).

A.



B.



C.

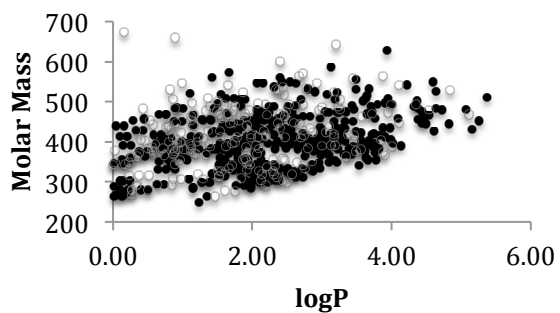


Figure 8. Mass and LogP values of 5-NI Compounds by Strain

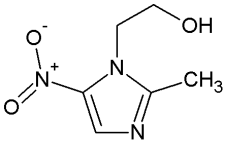
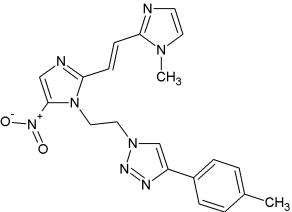
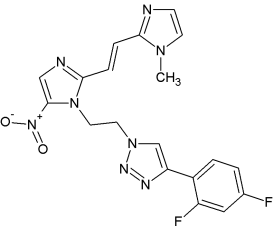
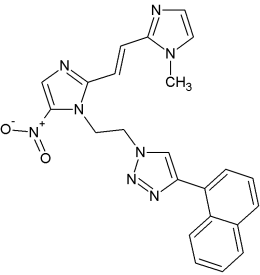
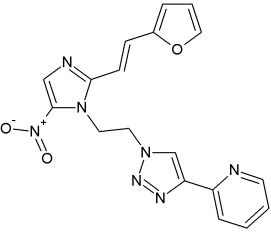
(A) Strain SS1. Each empty gray circle represents a compound that was either less potent than Mz or produced a pEC50 value equal to 4.7. Each black circle represents a compound more potent than Mz.

(B) Strain CS22

(C) Strain 1.5R41B

We found that active compounds do not fall within a set range of logP of molar mass values in Mz-sensitive strains. No general statement about the relationship between a compound's potency and its molar mass or logP values could be made for Mz-resistant strains since there were fewer compounds with measurable pEC50 in those strains. Active compounds in strain RdxA:FrxA, which were limited to its top eight most potent compounds, did share similar physical features. These compounds had a logP that value ranged between 2.75 and 4.25 and a molar mass that was between 440 and 360 (g/mol). Interestingly, all broad-strain compounds fit molar mass and logP criteria for active compounds in RdxA:FrxA (table 2). However, only 4.3% of all compounds that fit the same criteria were actually broad-strain compounds.

Table 2. Physical Properties of Mz and Broad-Strain Compounds

Name	Structure	MM (g/mol)	logP
Metronidazole		171.15	-0.46
Jar-3-215h		404.43	3.40
Jar-3-215d		426.38	3.17
Jar-3-214g		440.46	3.87
Jar-3-210i		377.36	2.78

These results suggest that molar mass and logP cannot be used to predict if a compound will be active in Mz-sensitive strains. We also found that even though broad-strain compounds fall within a certain range of logP and molar mass values it does not mean that compounds created to fall within those values will also be “broad-strain”. While those logP and molar mass values are

required for compounds to be broad-strain they are not sufficient to accurately predict if the compounds will be active across multiple strains.

Relationship Between a Strain's Mz sensitivity and Alkyne Contribution

All compounds screened are made up of a core unit and an alkyne unit. The alkyne unit is made up of an alkyne group attached to one of 110 different substituents. Those alkyne units are the main source of chemical diversity found in the 5-NI compounds. With abundant alkyne unit diversity we asked if the structure of an alkyne unit could be used to determine if a compound would be potent in Mz-sensitive versus Mz-resistant strains? This information would not only aid in drug design but would also be helpful in determining the structural configuration of drugs targets. In Mz-resistant strains "top alkynes" are the six alkynes with the largest average potency values, in Mz-sensitive strains "top alkynes" are the twenty alkynes with the largest average potency values. "Top alkynes" were selected by taking the average potency for all six compounds with the same alkyne unit, so these values are actually averages of compounds with the same alkyne unit but different core units. The top 20 alkyne units from both Mz-sensitive strains were compared first (figure 9).

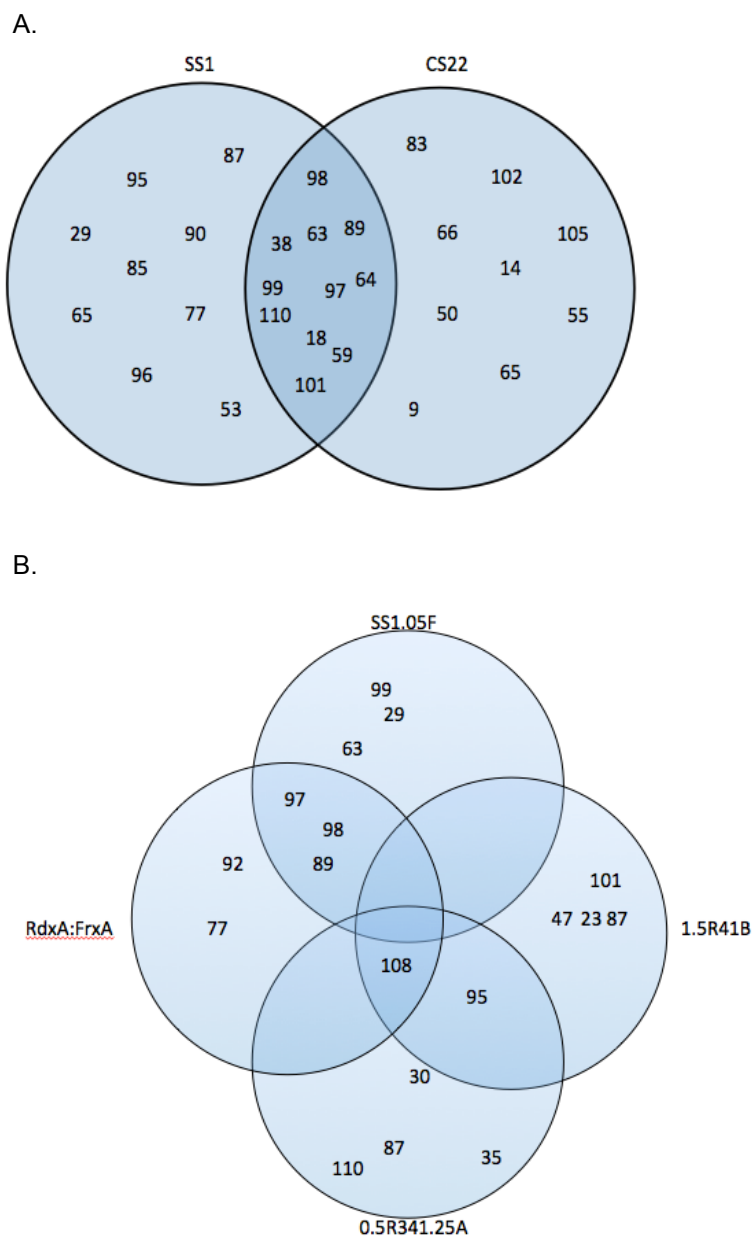


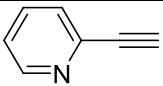
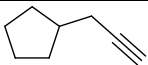
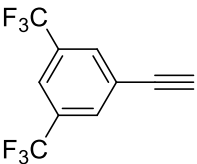
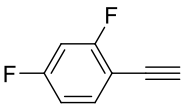
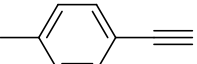
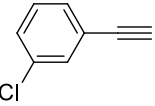
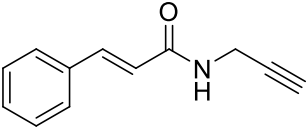
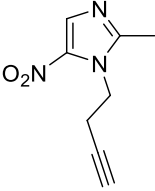
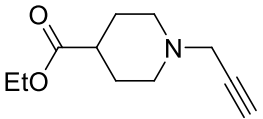
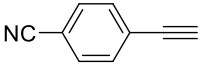
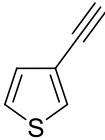
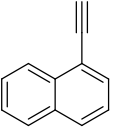
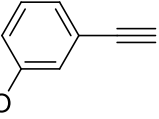
Figure 9. “Top Alkynes” Shared by Mz-Sensitive and Mz-Resistant Strains

(A) Venn Diagram Comparing Top 20 Alkynes Shared Between Mz-Sensitive Strains SS1 and CS22. Each number corresponds to an alkyne. The number was determined by taking the average potency of compounds with the respective alkyne. Compounds in overlapping section represent alkynes with “top” potency in more than one strain.

(B) Venn Diagram Comparing Top 6 Alkynes Shared Between Mz-Resistant Strains SS1.05F, 1.5R41B, 0.5R341.25A, and RdxA:FrxA.

Both strains shared eleven of the top 20 alkyne units found in each strain. Apart from the presences of a cyclic ring, which ranged from benzene to triophene, the eleven alkynes did not share any other structural features (table 3).

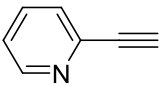
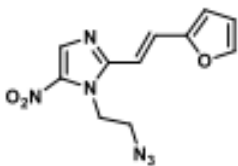
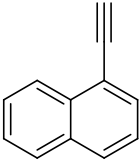
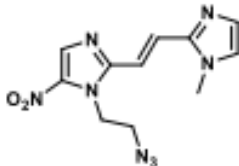
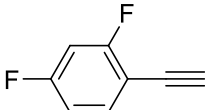
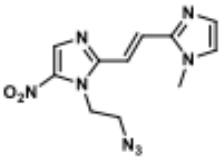
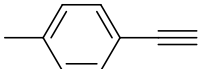
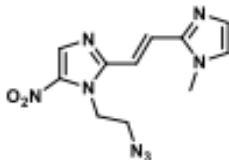
Table 3. Top Alkynes in More Than One Strain

Alkyne Number	Alkyne Structure	Alkyne Number	Alkyne Structure
108	 Molecular Weight: 103,12	64	 Molecular Weight: 108,18
95	 Molecular Weight: 238,13	97	 Molecular Weight: 138,11
101	 Molecular Weight: 116,16	99	 Molecular Weight: 136,58
59	 Molecular Weight: 185,22	38	 Molecular Weight: 179,18
18	 Molecular Weight: 195,26	63	 Molecular Weight: 127,14
110	 Molecular Weight: 108,16	89	 Molecular Weight: 152,19
98	 Molecular Weight: 132,16		

Three of the four Mz-resistant strains shared alkyne unit 108. Two of those four strains shared alkyne units 97,89,89, and 95 not all alkyne units were not shared by the same two strains. Out of the eleven alkyne units shared by SS1 and CS22, seven were also a top alkynes

in at least one Mz-resistant strain. The three top alkyne units shared between SS1.05F and RdxA:FrxA were also shared between the top alkynes in CS22 and SS1. All four alkynes units found in the broad-strain compounds are top alkynes in at least 3 different strains. Out of the four broad-strain compounds two (Jar-3-214g and Jar-3-215d) contained top alkynes from at least four different strains. Interestingly, compound Jar-3-210i contained alkyne unit 108, which was the only top alkyne found in three of the four Mz-resistant strains but was not found in the top twenty of either of the Mz-sensitive strains. We found that 63% of the top alkyne units shared between the two Mz-sensitive strains were also top alkynes in at least one Mz-resistant strain. That led us to believe that it was not possible to use alkyne unit structure to predict if a compound will be potent in an Mz-sensitive versus and Mz-resistant strain. This finding is supported by the observation that 75% of the alkyne structures attached to the broad-strain compounds are top alkynes in both Mz-sensitive strains and two of the four Mz-resistant strains (table 4).

Table 4. Alkyne and Core Structure of Broad-Strain Compounds

Compound	Alkyne	Core
JAR-3-210i	 <p>Molecular Weight: 103,12 108</p>	 <p>Molecular Weight: 274.08 10.3mg, 0.0375mmol</p>
JAR-3-214g	 <p>Molecular Weight: 152,19 89</p>	 <p>Molecular Weight: 288.11 10.8mg, 0.0375mmol</p>
JAR-3-215d	 <p>Molecular Weight: 138,11 97</p>	 <p>Molecular Weight: 288.11 10.8mg, 0.0375mmol</p>
JAR-3-215h	 <p>Molecular Weight: 116,16 101</p>	 <p>Molecular Weight: 288.11 10.8mg, 0.0375mmol</p>

Core Chemistry is Predictive of Compound Potency Across Multiple Strains

While there are 110 different alkyne units there are only six different core units. The core unit contains the nitro group that is hypothesized to be responsible for the killing effect of 5-NI compounds. The core of every compound contains the nitro group in the same location. However, the location of three other substituents varies between the six different core units. With much less structural variation between the six core units compared to the 110 alkyne unit we asked what configuration of core substituents was associated with, on average, the most potent compounds in each strain? To investigate this question we sorted the compounds according to their core structure and we found the average potency of all the compounds with the same core (figure 10)

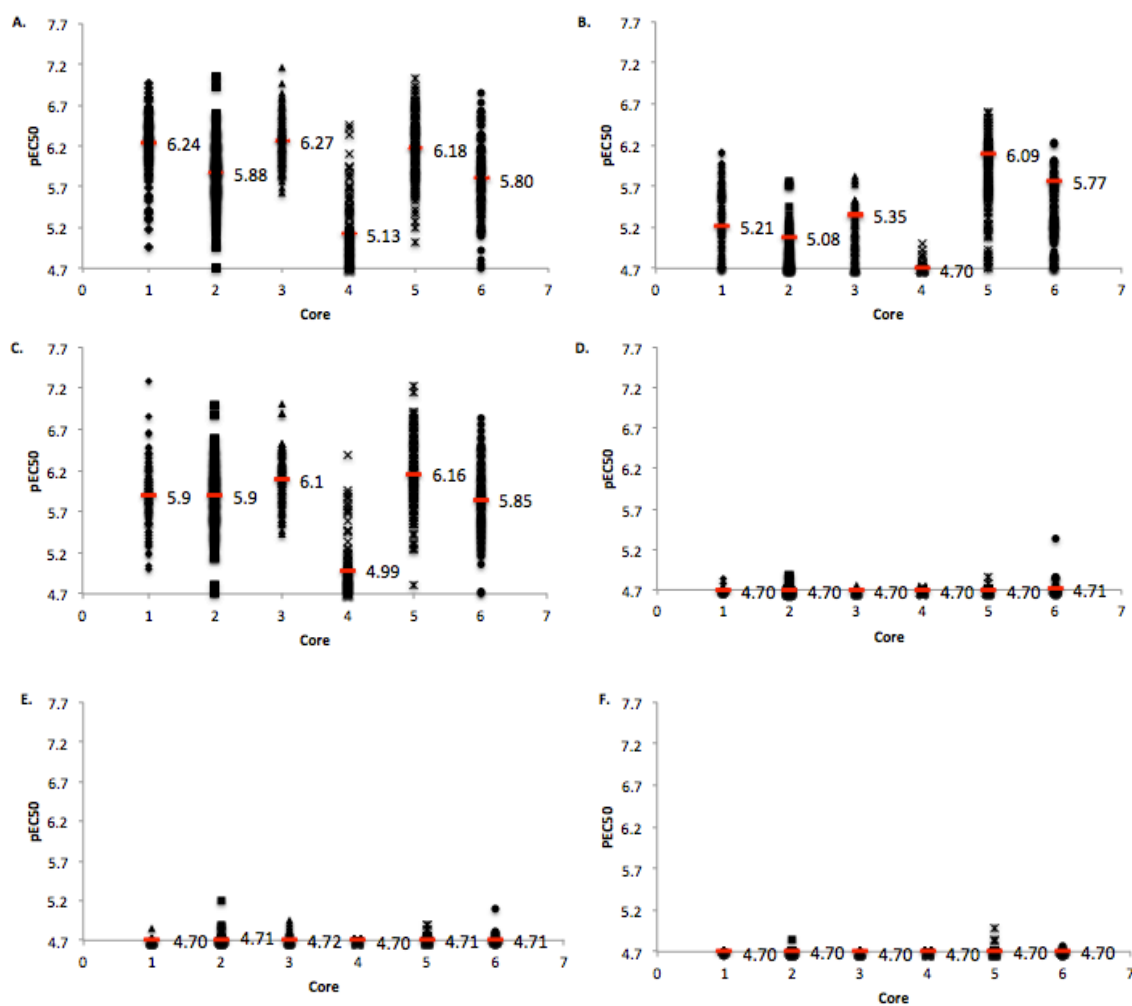


Figure 10. Average Core Potency By Strain

(A) Strain SS1. The number to the right of the red line represents the average potency of all compounds from the representative core.

(B) Strain CS22

(C) Strain SS1.05F

(D) Strain 1.5R41B

(E) Strain 0.5R341.25A

(F) Strain RdxA:FrxA

Core 4, on average, contained the least potent compounds across all *H. pylori* strains.

The 4 position on core 4 is an ethyl group connected to an azide, this is the location where the alkyne unit would join the 5-NI ring. All other core units have the azide on either the 1 or 2 position and hydrogen in position 4 instead. Compounds synthesized with core 4 were clearly the

least potent across all strains tested. While compounds with core 3 are the most potent in SS1 and 0.5R3241.25A, compounds with core 5 are the most potent in CS22 and SS1.05F, and compounds with core 6 are the most potent in 1.5R41B. On average, there was no core associated with higher compound potency in RdxA:FrxA. There was not a single core that was the most potent across all strains. However, core units 5 and 6 are structurally similar except that core 6 contains an imidazole in the 2 position of the 5-NI ring while core 5 contains a furan in the same position. Unlike core units 5 and 6, core unit 3, which was the most potent core in two strains, had its azide group on the position 2 instead of position 1. Also, core 3 had a methane group instead of a ring group in position 1. While we did not find a structural configuration that was associated with the most potent compounds across all strains we did find that, in general, a 5-NI compound with the azide in position 4 were associated with the least potent compounds in all strains we screened.

Using a Compound Activity Profile as a Tool to define Mz-Resistance Mechanisms

Finally, we wondered if there was a way to use a strain's compound activity profile as a way to gain a better understanding of Mz-resistance mechanisms. In the same way that sequencing an organism's genome can be used as a method of classification we believe that the 5-NI compound activity profile of *H. pylori* can be used to sort it based on its Mz-resistance mechanisms. Correlation coefficient analysis is one method that uses a strain's individual activity profile as tool for grouping strains based on their sensitivity to 5-NI compounds. However, the limited number of compounds with measureable pEC50's in the most Mz-resistant strains makes it difficult to attain significant results for comparisons of correlation coefficients. In order to investigate the relationship between the six strains we used the Neighbor program from a computational phylogenetic package called Phylip. Neighbor takes a distance matrix, where the values in the matrix represent the difference between the pEC50 of the same compound in two different strains, to create a tree (figure 11).

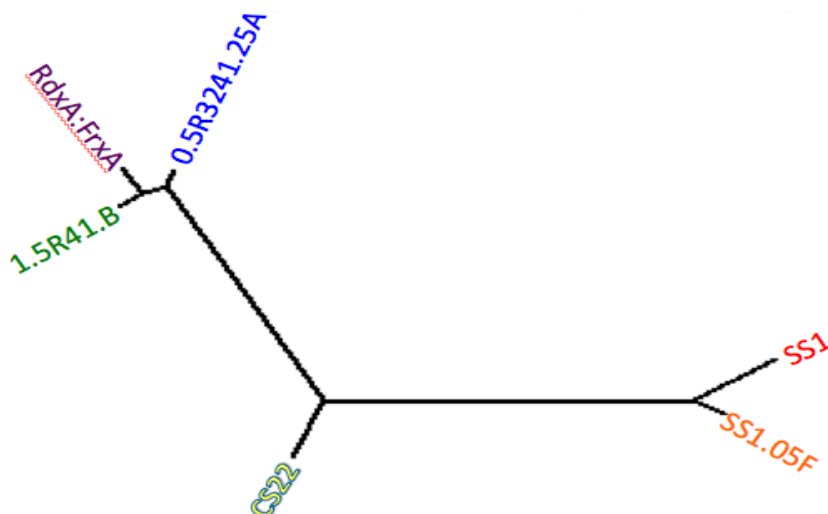


Figure 11. Nine-Compound Tree

The position and distance of the tree's branches correspond to the degrees of similarity between the different strains in regard to the way they respond to the same compounds. In the distance matrix we used nine compounds that produced measurable pEC₅₀'s in all six strains. The tree revealed that the strains grouped in three distinct locations. At the end of one branch there were three strains 1.5R41B, RdxA:FrxA, and 0.5R3241.25A at the furthest end from that group was another branch with two strains SS1, and SS1.05F. In between the two groups was CS22. The tree branching suggests that the strains use three different mechanisms to avoid activating 5-NI compounds and implies that Mz-resistance occurs in multiple steps.

DISCUSSION

Expanding upon earlier research on the use of 5-NI compounds as new drugs candidates in parasites our lab tested the activity of these compounds in *H. pylori*. We have shown that 5-NI compounds have the ability to inhibit bacterial growth at concentrations smaller than are required by Mz. However, limitations in the sensitivity of the screening protocol, based on issues with compound solubility at high concentrations, have resulted in the lack of potency values for most of the compounds screened in the Mz-resistance strains. We found four compounds that are not only active in geographically distinct Mz-sensitive strains of *H. pylori*, but are active also in Mz-resistant strains. Screening experiments also revealed physical and structural characteristics that could be used to design new 5-NI drugs in the future. Finally, we suggest that a strain's compound activity profile can be used as a tool to gain a better understanding of Mz-resistance mechanisms in *H. pylori*.

Overcoming Mz-Resistance

Our research on the utility of 5-NI compounds as antibiotics has focused on finding compounds that are not only more potent than Mz, but also more likely to overcome Mz-resistance. While it is straightforward to compare the potency between two compounds by measuring their pEC50 values, measuring the ability of a compound to overcome antibacterial resistance is not as straightforward. In the case of *H. pylori* and Mz treatment, a compound is able to overcome one of the principle bacterial resistance mechanisms, the inactivation of nitroreductases, in one of two ways. It can act through a mechanism that does not involve activation by a particular nitroreductases, causing any changes in the functionality of those nitroreductases to become irrelevant. The second way a compound can overcome Mz resistance is by being more potent than Mz. In this situation a compound can saturate any nitroreductase activity present and exert its killing effect. What makes the second case possible is that nitroreductases have a natural role in *H. pylori* physiology and eliminating them completely from the cell might lead to a fitness cost for the strain. Examples of decreased cell growth in antibiotic

resistant strains can be seen in Clarithromycin resistant strains of *H. pylori* (Britta Bjorkholm 2001). 5-NI compounds that are more potent than Mz in the SS1 are probably activated by nitroreductases in the bacteria. However, any compound that is more potent than Mz in the double mutant must have been activated by a nitroreductase other than RdxA or FrxA. This indicates that there are other nitroreductases playing a role in 5-NI compound activation. It also means the new candidate compounds were able to overcome the Mz-resistance mechanisms of an *H. pylori* strain that was engineered to be resistant to 5-NI compounds. To measure the fitness cost of Mz resistance in *H. pylori* the growth rate of SS1 and RdxA:FrxA should be compared.

Likelihood New Compounds Are Orally Active in Humans

We investigated the druglikeness of the four broad-strain compounds using Lipinski's rule of five. Meant to serve as a rule of thumb for predicting if a compound has the pharmacological properties required to make it orally active in humans, Lipinski's rule of five, incorporates the molar mass, logP, the number of hydrogen bond donors, and the number of hydrogen bond acceptors of a compound. The rule states that, in general, compounds that are orally active do not violate more than one of the five rules. All four compounds we found met Lipinski's five criteria for an orally active drug (table 5).

Table 5. Lipinski's Rule of Five Analyses of Four Broad-Strain Compounds and Mz

Compound	Molar Mass	LogP	Number of H-Bond Donors	Number of H-Bond Acceptors	Passes Lipinski's Rule of Five
Jar-3-210i	377.36	2.78	0	10	yes
Jar-3-214g	440.38	3.87	0	10	yes
Jar-3-215d	426.38	3.17	0	10	yes
Jar-3-215h	404.43	3.40	0	10	yes
Metronidazole	171.15	-0.46	1	4	yes

The next step for the candidate compounds is to study their ability to clear an *H. pylori* infection *in vivo*. Also, it is important to determine the toxicology of the compounds before they are subjected to further trials.

Broad-Strain Compounds Share Specific Physical Properties and Chemical Structure

After screening more than 600 compounds against six strains we have collected enough data to draw conclusions about the physiochemical and structural features of 5-NI compounds that should be emphasized and avoided in future drug design. Our finding that neither the logP nor the molar mass of a compound is indicative of compound activity in Mz-sensitive strains means that if we want to use a compound's physical characteristics to predict its activity in *H. pylori* we need to use something other than its logP or molar mass. Yet, those two values are important in relation to the druglikeness of a compound so, the values should be taken into account but should not be used when attempting to predict of a compound's activity. While we found that no specific alkyne unit or core unit was predictive of potency in all strains screened we did find that 5-NI compounds with an azide instead of hydrogen in position four of the 5-NI core unit were associated with compounds with the least average potency in all strains tested. Going forward, these experiments suggest that new compounds should avoid having a 5-NI core similar to core 4.

Tree Analysis of 5-NI Compounds a Tool for Analysis of Mz-resistance Mechanism

The exact mechanisms of Mz resistance in *H. pylori* are still a topic of debate. Using the potency values of select compounds we were able to show that six different strains branched into three different groups. Our laboratory has five *H. pylori* clinical isolates from all over the world with unknown compound activity profiles. Screening these strains with the same nine compounds already used on the six other strains would give the tree more definition. Going a step further and sequencing the genomes for all the strains used in the screening experiments and analyzing the *rdxA*, *frxA*, and *fdxB*, genes would provide a method to verify if the different branches in the tree actually correspond to different patterns in nitroreductase functionality. Being able to use the 5-NI compound's as a tool to decipher a strain's Mz-resistance mechanism is important because it can allow us to quickly and easily gain a better picture of antibiotic resistance in *H. pylori* apart from genetic sequencing.

Future Directions

Our work has expanded the number of organisms that show sensitivity to 5-NI compounds. It would be interesting to test the activity of the four broad-strain compounds on the two parasites, *G. lamblia* and *T. vaginalis*, which are already known to be sensitive to 5-NI compounds (Jacqueline A. Upcroft 2006). Just as Mz is active in numerous microorganisms perhaps one of the four compounds we have found has the potential to work across multiple species as well. The existing compound library can also be used to screen against neglected tropical disease that are known to be susceptible to 5-NI compounds. For example, Chagas disease, caused by *Trypanosoma cruzi* is sensitive to the 5-NI derivative Fexinidazole (Bahia MT). Perhaps one of the 660 new 5-NI compounds we already screened against *H. pylori* is a future treatment for that disease. Overall, much remains to be done in order to reach our goal of transforming our research on new 5-NI drugs into clinical applications. This work is especially important for people with low socioeconomic status who are most likely to be infected with *G. lamblia*, *T. vaginalis*, *T. cruzi*, and *H. pylori*. Since pharmaceutical companies have found that curing disease like Giardiasis, Chagas, and Ulcers are not economically advantageous, research and development of new treatments for infectious disease must come from laboratories like ours, and others, around the world.

METHODS AND MATERIALS

***H. pylori* culture conditions**

All *H. pylori* strains were grown in Bacto Brain Heart Infusion media (BD) supplemented with 0.4% IsoVitaleX (BBL), 10% Fetal Bovine Serum (Omega) and, Amphotericin B (8 µg/ml). Bacteria were incubated in a 25 cm³ flask in microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) created with a Pack-MicroAero (MGC) at 37° for 24-72 hours. BHI agar plates were made using Brain Heart Infusion agar (Criterion) with 0.4% IsoVitaleX (BBL), 10% Horse blood and, Amphotericin B (8 µg/ml).

Laboratory derived Mz-resistant *H. pylori* Strains

All three Mz resistant strains were created using SS1 as the parental, Mz-sensitive, strain. Strain 0.5R321.25A was initially grown on a BHI blood-agar plate with a Mz concentration of 0.5 µg/ml a single colony from that plate was expanded in liquid BHI media and was then plated on a BHI blood-agar plate with 1.25 µg/ml of Mz. Strain 1.5R41B was established by growing SS1 on BHI blood-agar plate with 1.5 µg/ml of Mz and picking a single colony. Strain SS1.05F was isolated from a BHI blood agar plate with 0.5 µg/ml of Mz. After each of the strains was isolated they were grown in BHI with additives without Mz.

Screening

Compound plates with 10 mM compounds were diluted to 300 µM in a 96 well plate, the last row of the compound plates for strains SS1, RdxA;FrxA, SS1.05F, 15R41B, 0.5R3241.25A had 300 µM Mz the compound plate for CS22 had 300 mM Mz in the last row. The compounds were diluted to their final concentration in BHI that had not been supplemented with any additives. Using Biomek Software a Biomek 3000 Automated Workstation assisted in executing ten 1:3 serial dilutions in the experimental plate, the most concentrated well had a concentration of 20 µM. Column 1 and 12 of the experimental plate did not contain compound. While only columns 2-12 were inoculated with *H. pylori* at a 1:5 dilution. The OD₆₀₀ of the initial inoculum was between 0.30-0.50 and was diluted in BHI, with additives, to 0.06-0.08 before it was added to the wells. After between 48-72 hours of incubation in microaerophilic conditions at 37° C the OD 600 of the

96 wells were read with a spectrophotometer (SpectroMax M2^e) and collected with SoftmaxPro 5.2 software.

Computational Analysis of Raw Screening Data

In order for the data to be valid the OD 600 of the cultures had to be greater than 0.75. The wells in the column number 1 are the negative control for the experiment, while the wells in column number 12 are the positive controls. The last row of the 96 well plate is an experimental control which always has Metronidazole. The raw data from the 96 well plate was uploaded to a program called BioAssay, developed by CambridgeSoft, which fits a dose-response curve to the data using a non-linear regression model. In order for the data to be valid it must have an R² larger than 0.88 if it has an EC50 smaller than -4.7.

Statistical Analysis

Pearson product-moment correlation coefficient, r , was determined in the comparison of the compound activity profile between the 6 different strains. Where X and Y represent the two different strains. The formula to determine r is as follow:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

The percentage probability that an uncorrelated r would be larger than the r determined depends on the number of pair data sets.

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